# Studies on Mineral Oil Antigen Adjuvants

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# Part One

# INTRODUCTION

## A. THE ADJUVANTS

The adjuvants, non-specific substances stimulating the development of immunity, have been developed, mainly empirically, in response to a need for methods which would simplify and extend the possibilities of vaccine therapy. Jenner's and Pasteur's convincing demonstrations of the effectiveness of artificially created immunity opened up a vast and exciting field; but in practice the low antigenicity of some materials, the short persistence of detectable antibodies and the need for repeated injections, often hindered its full application in the field of prophylaxis.

The rapid development of techniques for the study of humoral immunity which followed Von Behring and Kitasato's discovery of antitoxin in 1890, led many workers to investigate the factors which governed the development of serum immunity. To obtain something for nothing is always attractive, and the adjuvant effect received a fair share of attention. Not, however, until Ramon's and Glenny's publications of 1925 and 1926 did any of the discoveries made lead to their immediate practical exploitation as an adjunct to immunization procedures.

The term 'immunologic' or 'antigen adjuvant' is probably best reserved for substances which can be given mixed with the antigen and which have no other gross physiological properties at their effective dose level. The first substances shown to have an adjuvant action were, however, already known to have a general stimulant action on the metabolism of the body.

In 1904 Friedberger injected rabbits with alcohol and at the same time gave them a heat killed cholera vaccine (Vibrio metschnikovii?) intravenously. After a single dose of alcohol they produced up to four times as much antibody as did controls. Others, given repeated injections of alcohol, showed a twenty-five fold increase. Friedberger also appears to have been the first to notice that an adjuvant effect is obtained when Gram-negative bacteria are included in a mixed vaccine. The addition of heat killed typhoid bacilli to his cholera vaccine, gave up to 37 times more antibody against the latter.

Nothing seems to have come of these observations at the time, and the next reports are those stemming from the work of Agazzi in 1909. He showed that very small doses of arsenic compounds enhanced the agglutinin production of

rabbits against Bacterium typhi-giessen. This effect was confirmed by Friedberger and Masuda in 1911 who showed up to 32 fold increases in antibody production by rabbits against V. metschnikovii, typhoid bacteria and sheep red cells when single doses of Salvarsan were given at the same time.

Also in 1911, Hektoen was the first of a number of workers to report on the adjuvant action of sodium iodoxybenzoate and similar compounds whose oxygen content was thought to be physiologically active. He found that a single dose of this compound stimulated a tenfold increase in peak antibody titres in the dog against goat red blood cells.

In 1916 (a), Le Moignic and Pinoy produced the first 'lipovaccines' and showed that mineral oil might be useful not only as an excipient for vaccines but also as an adjuvant. The subsequent development of oil adjuvants will be discussed in detail below; the immediate effect of Le Moignic and Pinoy's work was the widespread use of oils as convenient vehicles for various antigens, rather than the exploitation of their adjuvant effect.

Strong incentives for a search of the laboratory shelves for substances stimulating

antibody production were Ramon's first report on the use of tapioca in 1925 and Glenny et al.'s exciting discovery of the effect of alum in 1926.

Ramon reported on 7th May 1925 that horses developing abscesses at the injection site during routine hyperimmunization with diphtheria toxoid, developed much higher antitoxin titres than those without. He found that the deliberate infection of the inoculation site with streptococci and staphylococci produced the same effect, but that there was no increase when an abscess was provoked away from the site. Ramon experimented with various materials with the object of producing a sterile inflammatory lesion and eventually found that tapioca was simple to use and very effective.

Glenny, Pope, Waddington and Wallace (1926) were attempting to purify diphtheria toxoid by precipitation with potassium alum and other substances. They found that toxoid precipitated by acetic acid was still an effective antigen, and was in fact a slightly better one than the fluid toxoid. This discovery led them to test the antigenicity of the alum precipitate itself rather than the purified eluate from it. This precipitate turned out to be strikingly more antigenic than the fluid toxoid from which it was

prepared. In this way the foundation was laid for the present extensive use of vaccines containing alum, and aluminium hydroxide precipitated antigens of all kinds.

Since 1925 an enormous number of miscellaneous substances have been added to antigens in the hope of producing an adjuvant effect, mostly with little rationale except to follow Ramon and his tapioca in trying to absorb the antigen or cause some inflammation at the site of injection. The two pioneers were at the forefront of this work. In 1926 Ramon reported that gelatin was effective and turpentine or toluol, causing tissue damage, were found by Glenny and Waddington (1928) to produce an increase in antigenic efficiency similar to that produced by tapioca. Glenny et al. (1931) tested cerium nitrate, zinc sulphate, calcium chloride, tungstic acid and dialysed iron as precipitants for diphtheria toxoid. They all had an adjuvant effect. Calcium chloride was added to diphtheria toxoid and tested in children by Ramon and Nélis (1932); the calcium chloride became converted to calcium phosphate so that the procedure was not as heroic as it sounds, though repeated injections caused some violent general reactions. Ramon, Lemetayer and Richou (1937)

found that saponin caused tissue damage and scarring but fair adjuvance for diphtheria toxoid, and again Ramon et al. (1949) showed that even pectine and glycerine had some stimulating effect on antibody production against tetanus toxoid.

Many substances which were tried did not produce any adjuvant effect; those which do are a strange miscellany. Lewis and Loomis (1926) found trypan blue effective in increasing the production of haemolysin against sheep erythrocytes. Nemchiloff (1937) reported on the adjuvant action of charcoal, cholesterol, and codliver oil. Boquet and Sandor (1937) found bread crumbs, infusorial earth and egg yolk effective adjuvants and Ungar (1952) mentions the use of yeast cells. Dresser (1961) showed that sorbital mon-oleate (Crill 16), sorbital monolaurate (Span 20) and oleic acid possessed some stimulating effect in antigen elimination tests, and Sakanyan et al. (1960) used caffeine to increase antibody production to live Brucella abortus vaccine. Pernis and Paronetto (1962), after suggestions that some immunologic mechanism might be involved in the pathogenesis of silicosis, showed that silica could be used as an adjuvant.

Attempts to adsorb the antigen to some substance so that a depot is formed at the injection site have become more sophisticated in recent years. Adsorption on to the latex of Hevea brasiliensis (cultivated rubber) (Jacotot, 1947) or collodion particles (Ungar, 1952) are effective methods. Noll and Youngner(1959) found that influenza virus was strongly and irreversibly adsorbed to hexdecylamine and lost its infectivity. Guinea-pigs given the adsorbed virus produced about ten times more haemagglutinating antibody than controls. Even plastics have been used, anthrax antigen adsorbed on to styrene crosslinked by antibody was shown by Kent and Slade (1960) to be more antigenic than alum precipitated antigen in sheep, rabbits and guinea-pigs. A clever method of fixing the antigen in one place is that of Amies (1959) who mixed sodium alginate with it. This becomes converted to insoluble calcium alginate after subcutaneous injection and the adjuvant effect is similar to that of aluminium phosphate. More subtle still is the action of phosphorylated hesperidin. This substance, which inhibits the action of hyaluronidase (Preston et al. 1953) is used as an adjuvant for drugs (Cohen et al. 1953). Martin, Moss and Beiler (1953) found that it also

acted as an antigen adjuvant in rabbits given typhoid vaccine and they later showed that it had an adjuvant action, possibly comparable with that of water-in-oil emulsions, when used with influenza virus vaccine, tetanus and diphtheria toxoids, and pertussis vaccine (Moss, Beiler and Martin, 1956).

An adjuvant action is shown by some drugs and physiological effects that, whilst not holding out much hope of practical application, may be of value in helping to elucidate the mechanism of adjuvance. For instance, sensitivity to horse serum was shown by Swift and Schultz (1936b) to potentiate antibody formation against lens extracts in the rabbit. Good et al. (1957) confirmed this by showing that rabbits, already sensitized to tuberculin, produced more antibody against an antigen injected at the same time as a test dose of tuberculin than did unsensitized controls. Winter et al. (1962) found that if a course of treatment with cortisone or prednisolone was finished a few days prior to sensitization with antigen there is stimulation of antibody formation. This is probably due to a 'rebound effect', i.e. the rapid build up of a depleted cell population. An intriguing report along the same lines is that of Hadnagy (1963) who found

that a cytotoxic serum prepared in the dog and directed against rabbit spleen, had a stimulating effect on antibody production by the rabbit. Once again this is probably due to a rebound of regenerating cell population. Of the drugs and vitamins, Katitch et al. (1962) found that $B_{12}$ had an enhancing effect on tetanus antitoxin production. Long and Shewell (1955) found that thyroxine treatment increased the level of diphtheria antitoxin in the guinea-pig and Ungar in the same year reported that adrenalin, benzedrine and ephedrine stimulated antibody formation to diphtheria toxoid. Other amines without a vaso-constrictor action had no effect.

In practical vaccination procedures, vaccines adsorbed on to alum or similar materials, for long held the field. Few of all the long list of substances cited above have had any practical application, a strange exception being saponin which has for long been added to anthrax spore vaccine for cattle (E.P. Lindley, personal communication, 1960). The alarming finding (Hill and Knowelden, 1950) that inoculation with alum based vaccines might potentiate paralytic poliomyelitis; an impression amply confirmed by Bodian's experiments on monkeys reported in 1954, led to the near abandonment of this type of

adjuvant for use in man, though it is still extensively used in vaccines prepared for veterinary purposes.

The known stimulating effect of certain bacterial preparations on toxoidal immunity was exploited to take its place. Although Friedberger in 1904 had shown adjuvance in mixed vaccines, Ramon and Zoeller (1926) were the first to mix a toxoid (diphtheria) with a bacterial vaccine (T.A.B.) and show that the combination worked well. In 1927 they showed that T.A.B. had an adjuvant effect in man on responses to tetanus toxoid given at the same time, and in 1936 Bordet first used diphtheria toxoid mixed with pertussis vaccine to produce the adjuvant effect amply confirmed by later workers (Fleming et al., 1948).

This use of bacteria themselves or their products as adjuvants had already been investigated by various workers. Lewis and Loomis had found Brucella abortus and a streptococcal infection to be adjuvant (1926). Ramon, Richou, and Staub (1937) tested Preisz-Nocard bacilli and Ramon et al. (1949) killed Brucella abortus. The finding of Swift and Schultz (1936a) that staphylococcal toxin itself was an adjuvant is of significance in explaining the effectiveness of abscesses at the injection site

as first noticed by Ramon in 1925. The stimulating effect of Gram-negative bacilli appears to be due to their endotoxin content, the adjuvant effect of which was first noticed by Stuart and Windel in 1951 and then investigated in detail by Johnson, Gaines and Landy (1954 & 1956).

One of the most important observations was that of Lewis and Loomis (1924) who noted that living virulent tubercle bacilli given intraperitoneally at the same time as various antigens, caused enhanced antibody formation to them. This was exploited by Freund and McDermott (1942) in their outstanding work involving the combination of mycobacteria and oil to make the most widely used and useful adjuvant for experimental work at the present time. Subsequently it was found that Freund's adjuvant was still effective for many purposes even if the mycobacteria were left out. The stage was thus set for the development of oils as safe and effective adjuvants for the immunization of man.

## B. THE OIL ADJUVANTS

Le Moignic and Pinoy (1916a) appear to be the first workers to have used a vaccine in which the antigen was enclosed in mineral oil. Rabinovitsch in 1897 had incorporated mycobacteria into butter and injected the material into animals, but he was interested in the histopathological response as was Grassberger (1899) who found that paraffin oil was a more effective vehicle.

Le Moignic and Pinoy found that it was necessary to use lanolin as an intermediary for incorporation of salmonellae into the oil. They therefore produced a true water-in-oil emulsion, remarking that the bacteria are 'embalmed' in the oil. Vaccines made in this manner protected mice against challenge with S. paratyphi B. and S. typhimurium. In the latter case they reported that it was markedly more effective than a saline vaccine, but were mainly interested in the safety of the materials and the reduction of reactions. Olive oil was also tried but in their hands this caused abscesses, though Achard and Foix in the same year reported on agglutinin production by guinea-pigs against S. paratyphoid B incorporated in this oil. Achard and Foix did

not use lanolin as an emulsifier and their results show no adjuvant effect. Later, Le Moignic and Pinoy (1916b) reported a test in man of a triple vaccine of S. paratyphi A & B and Eberths bacillus (Salmonella typhi?), all incorporated in mineral oil. There were no local or general reactions and excellent titres were raised against all three antigens.

Whitmore, Fennel and Petersen (1918) incorporated various vaccines into lanolin and vegetable oils. They showed that a typhoid vaccine so prepared gave as high an agglutinin titre after one dose as did three doses of a saline vaccine. Cecil and Vaughan (1919) used a pneumococcus vaccine in vegetable oil extensively in American Army camps and apparently achieved some degree of protection. They reported that Whitmore, finding that olive oil caused some degree of subcutaneous irritation, now prepared the vaccines with cotton seed oil.

By 1934, these lipovaccines had fallen into some disrepute and Fleming was of the opinion that, although they did confer some immunity they were less effective than saline vaccines. Perhaps this was partly due to their use as vehicles for typhoid vaccine. Until very recently the protective value of all such

vaccines, however prepared, has been doubted. It was also due to the unfortunate circumstance that vegetable rather than mineral oils were used for preparing the vaccines. It is, perhaps, understandable that a bland natural oil should have been chosen rather than mineral oil with its irritant, inflammable, and carcinogenic relatives, but Saenz (1937), Friedwald (1944), Freund et al. (1948), and Berlin (1957) have all shown that there is very little adjuvant effect to be obtained from the vegetable oils.

The modern use of oil, linked closely with the name of Jules Freund as his 'complete' and 'incomplete' adjuvants (i.e. with or without mycobacteria), began with a chance observation by Freund et al. in 1937. They noticed that the complement obtained from guinea-pigs which had been given a single dose of killed tubercle bacilli in paraffin oil a year before, gave false positive reactions when used in complement fixation tests with tubercle bacilli. This turned out to be due to residual high antibody levels in the guinea-pigs, an unexpected finding as it is usually difficult to raise complement fixing antibodies against tubercle bacilli even by repeated injections.

It was known that the suspension of

Mycobacterium tuberculosis in solid paraffin or paraffin oil greatly favoured the development of tuberculin hypersensitivity (Coulaud, 1934; Saenz, 1935). The new observation of Freund et al. suggested that it might influence antibody formation as well.

M. tuberculosis itself has this effect on other antigens. In 1924 Lewis and Loomis had noticed that guinea-pigs infected with tuberculosis produced higher titres of antibody to unrelated antigens than did non-tuberculous pigs. They were able to show that living, virulent tubercle bacilli injected intra-peritoneally at the same time as an antigen (sheep red cells) caused enhanced antibody formation against it.

Dienes (1927; 1928) followed this up by showing that if the antigen was injected directly into a tuberculous lesion a similar effect was produced, together with a much more marked skin sensitization to the antigen, in this case egg-white. Dienes (1929) also showed that killed tubercle bacilli given at the same time as the antigen worked just as well. Later, Landsteiner and Chase (1940) suspended the dead tubercle bacilli in paraffin oil before injecting them intra-peritoneally with the antigen.

The critical contribution of Freund and

McDermott in 1942 was to incorporate the antigen into this mixture as well, by the use of an emulsifier promoting the formation of a water-in-oil emulsion.

Freund and McDermott's method was to mix the antigen (horse serum) with Aquaphor, a lanolin-like substance used as an ointment base. This was then blended with paraffin oil in which killed tubercle bacilli had already been suspended. Aquaphor or other emulsifiers were found to be essential for the formation of a stable mixture, as had been noticed earlier by Le Moignic and Pinoy. The mixture was found to sensitize guinea-pigs to horse serum without difficulty, and these animals also showed higher precipitin titres than did controls.

Freund's original mixture, his 'complete adjuvant' is widely used in experimental work with laboratory animals. It stimulates the production of high antibody titres and hypersensitivity against many antigens. It was soon found to be very useful for the production of allergic encephalomyelitis in guinea-pigs by a single injection of brain or spinal cord (Freund, Stern and Pisani, 1947) and in other studies of auto-immunity. It is also particularly useful in the examination of antigens for impurities by

raising detectable levels of antibody to minor components (Kabat, 1958).

Freund himself and other workers carried out experiments on immunization, with the help of complete adjuvant, against various diseases of man such as: Malaria (Freund et al. 1945), Influenza virus (Friedwald, 1944), and Shigella paradysenteriae (Halbert et al. 1946). Unfortunately, disastrous results followed the only recorded therapeutic use of a vaccine so prepared. Thousands of cattle in East Africa which were immunized with a formalinized rinderpest vaccine made up in complete adjuvant, developed an excellent immunity but also frank or hidden abscesses at the injection site. These led to condemnation of the carcases by meat inspectors at slaughter, or worse, their discovery in the butcher's shop (Scott and Ginsberg, 1959).

Killed Mycobacterium tuberculosis itself was not a component of the complete adjuvant for the above experiments, Freund and Walter (1944) and Friedwald (1944) having shown that non-pathogenic saprophytic mycobacteria such as M. phlei were just as effective for most purposes. The use of light, low viscosity mineral oils like Bayol F and the simple emulsifier Arlacel A

(mannide mono-oleate) simplified the preparation of the water-in-oil emulsions (Freund et al. 1948).

Long before the adjuvant rinderpest vaccine debacle, the marked local reaction to complete adjuvant had been noticed. Freund et al. (1940) even being of the opinion that the greater the reaction the greater the antibody production. Quite apart from other objections to the use of mycobacteria such as sensitization to tuberculin, these reactions made the adjuvant quite unsuitable for use in man and for most veterinary purposes.

The omission of mycobacteria (to form 'incomplete adjuvant') was shown by Freund and Walter as early as 1944 to cause little change in the ability of the oil to stimulate antibody production, though the potentiation of hypersensitivity was very much reduced (Freund, 1951). This incomplete form of the adjuvant was an obvious candidate for development as an adjunct to the immunization of man and domestic animals.

Tests were soon carried out by Henle and Henle (1945) who immunized human beings against A and B strains of influenza virus contained in a water-in-oil emulsion ('incomplete adjuvant'). Excellent titres of haemagglutination inhibiting

antibody were obtained, six times higher than those of controls given saline vaccine. There was also a persistence of the high titres in the adjuvant group for at least six months, but unfortunately nearly every subject developed a nodule at the injection site and some of these developed into abscesses.

Undaunted, Henle et al. (1946) continued with their investigations in man, showing that with an adjuvant influenza virus vaccine good 'protective' antibody titres lasted for over a year from vaccination. Mudd et al. (1948a & b) tested, in man, Shigella paradysenteriae vaccine and Hemophilis pertussis vaccines prepared in the same way. In this case the adjuvant effect was disappointingly small and the local reactions severe. Most subjects retained, for 6 to 12 months, subcutaneous nodules which became tender and increased in size periodically. In 60 per cent. abscesses either ruptured spontaneously or had to be opened. Volunteers given a second dose of the pertussis vaccine developed a rapid focal inflammation at the site of the nodule and Mudd et al. interpreted this as a hypersensitivity reaction of the Arthus type. The only hopeful aspect of their report was that systemic toxic reactions to the shigella vaccine were

very mild in the adjuvant group.

Doubtless the incomplete adjuvant caused similar nodules, tissue destruction and abscess formation in laboratory animals, but this was easily overlooked under their hair, as such lesions are much milder than those produced by the complete adjuvant.

Despite these discouraging effects Salk and Laurent began to experiment with an adjuvant influenza virus vaccine in 1952. They used a more highly purified oil and an emulsifier of a batch selected as causing no tissue reactions. They gave the completed vaccine intramuscularly instead of subcutaneously. The first tests were done in monkeys which showed maximum antibody titres 300 times higher than those of controls given the saline vaccine alone, and these high titres declined only slightly over a period of 4-12 months. The intramuscular route seemed to eliminate local reactions, there was no inflammation at the injection site and small doses could not be traced on histological examination.

These results were so encouraging that the vaccine was tested in man on a large scale. Salk, Bailey and Laurent (1952) reported that no local reactions occurred following the intramuscular injection of 2000 doses. There was

marked enhancement of antibody formation, and a good persistence of antibody for two years after vaccination (Salk, 1953).

Salk's results were the turning point for water-in-oil emulsion vaccines. Once he had shown that they could be safely and effectively administered to human beings the way was open for more extensive testing and the improvement of methods and materials.

A few cases of cyst formation still occurred after their use (Bell et al. 1961). This was traced to a particular lot of the Arlacel A emulsifier, so tests were worked out to screen batches for safety (Berlin, 1962). A more refined and tested oil (Drakeol 6 VR) was substituted for Bayol F, and by 1961 over 100,000 doses of adjuvant influenza virus vaccine had been given at Americal Naval and Air Force bases without mishap (Davenport, 1961).

That these vaccines were protective as well as raising high antibody titres was shown by Meiklejohn (1962) who obtained a 17:1 protection ratio against influenza strain A2 in men at an American Air Force base, though his tests were controlled by placebo rather than by the saline form of the vaccine.

Early tests in the United Kingdom were

unsatisfactory as nodule formation and late abscesses followed the injection of influenza virus vaccine in oil adjuvant (M.R.C. Report, 1957). In later trials, however, Himmelweit (1960) used toxicity tested oil and emulsifier and inoculated the vaccine intramuscularly. No local or general reactions were encountered and excellent levels of antibody were raised. Himmelweit also described a power emulsifier suitable for the large scale production of water-in-oil emulsions, which was a great advance on the laborious 'squirting through syringes' method previously used.

All these reports laid the basis upon which the world's first commercial, water-in-oil adjuvant vaccine for human use, was marketed in this country in 1963. This was 'Admune', an influenza virus vaccine prepared by Evans Medical Ltd. After forty-seven years of effort the mineral oil adjuvants had really arrived.

## C. THE MODE OF ACTION OF THE ADJUVANTS

Investigations into the mode of action of adjuvants divide broadly into two streams: the first dealing with adjuvants for which some physiological action on the antibody forming tissues can be postulated, i.e. the endotoxins, cortisone, etc.; and the second, those adjuvants forming an antigen depot at the site of injection, for instance the alums, and the oil emulsions. This thesis is mainly concerned with the last of these adjuvants.

The principal theory of the action of depot-forming adjuvants was formulated by Ramon in his first paper in 1925. Ramon suggested that the oedema, inflammation and collection of leucocytes at the injection site of tapioca absorbed vaccine, fixed the antigen in place, protected it from rapid destruction and allowed its gradual release into the circulation. Glenny *et al.* (1926) subscribed to this theory but also considered that the purity of their alum precipitated product and hence the absence of competing antigens might be of importance. Later, Glenny and Barr (1931) formulated the depot theory in a more precise form, suggesting that the slow release of antigen from a depot supplied repeated secondary

stimulations to the antibody forming apparatus and that these explained the high titres reached.

Slow release of antigen from the depot was held to apply also to the water-in-oil emulsions (Freund, 1947) and this theory is widely accepted as explaining the action of all adjuvants which form a depot at the injection site.

Direct support for the theory has come from a number of workers. Farago (1935) found that the longer a depot is left *in situ*, the longer is a high titre sustained, and the earlier a depot is removed the earlier the fall in titre starts. He also found that the peak titre is lower if the depot is removed before a maximum is reached. Ramon (1938) noted that the toxicity of highly poisonous substances such as tetanus toxin and strychnine could be greatly reduced by absorption into tapioca or incorporation into an oil emulsion. Halbert *et al*. (1946) found that active antigen was present in water-in-oil emulsion for up to 22 weeks after injection, and Herdegan *et al*. (1947) found that deterioration of *Shigella paradysenteriae* antigen in the depot at the site of injection was associated with a fall in agglutination titres though the oil emulsion itself was stable throughout.

The effectiveness of phosphorylated

hesperidin (a hyaluronidase inhibitor which prevents the rapid spread of an antigen away from the injection site) as an adjuvant lends considerable support to this theory (Moss et al. 1956). Finally, the findings of Parks et al. (1961) that the injection of antigen into the cornea or vitreous humour was as effective as intramuscular injection of the antigen after its incorporation into water-in-oil emulsion, seems good evidence that a slow release mechanism can be effective.

Although it is obvious that a depot of antigen is produced when these adjuvants are used and that for some time at least some leakage of antigen must occur, several workers have challenged the view that this is the only mechanism of their action.

Holt (1950) in a classic monograph on alum-precipitated diphtheria toxoid reported experiments showing that:

a) The repeated daily injection of plain antigen to mimic the slow release from a depot, did not produce an antibody level comparable to that seen after the alum-precipitated antigen had been used.

b) The removal of the nodule of antigen and adjuvant later than seven days after injection made no appreciable difference to the height of

the antibody response.

c) Four weeks after the initial injection, encapsulated depots were surgically removed. The reinjection of the material contained in these into the animals from which each depot had been removed, produced a strong secondary response. This suggested that by four weeks the depot had been completely walled off and was inactive.

d) Encapsulated depots, carefully dissected out unruptured from the subcutaneous tissue and then transferred to fresh guinea-pigs already sensitized to the antigen by a primary injection, produced only a tiny secondary response in them suggesting that any leakage of antigen was very small indeed and not enough to produce sustained high titres.

Holt considered that these results showed that the depot theory was inadequate.

Other experiments tending to show that the depot at the actual injection site was unimportant were reported by Popovici and Gogoaşă (1955) and Freund and Lipton (1955). Each of these reports described the removal of the depot between 1 hour and 48 hours after injection but this failed to affect the final antibody titres. Freund and Lipton were further able to show that there was a rapid dispersal of particles of

adjuvant (in their case a water-in-oil emulsion) from the injection site and that these reached the local lymph nodes within an hour of injection. This would vitiate the results of depot excision experiments.

Steiner et al. (1960) showed dissemination of Freund's complete adjuvant all over the rabbit's body following subcutaneous injection. Granulomata were found in the lungs, liver, kidneys and lymph nodes, a confirmation of the earlier findings of Rist (1937, 1938) who found that metastatic lesions in the lungs and lymph nodes appeared very soon after the subcutaneous injection of dead tubercle bacilli in paraffin wax or oil. Holt (1950) reported that very small doses of precipitated diphtheria toxoid formed no nodules and that the material was untraceable at post-mortem. He held this to imply that depots were not necessary for adjuvance but presumably small depots could have been formed in lymph nodes and other sites.

Another theory of the action of depot-forming adjuvants is that an antibody producing 'organelle' is formed round the adjuvant depot. Freund et al. (1952), when hyperimmunizing horses, found high titres of antibody in the fluid from areas of inflammation at the site of repeated

subcutaneous injections of diphtheria toxoid and oil adjuvant. The antibody concentration in this fluid was higher than that in the blood so they suggested that it was actually being formed at the injection site. Westwater (1940) had already noted that for the first three weeks following the injection of dead tubercle bacilli in oil, complement fixing antibody was more abundant at the injection site than in the serum. Salk et al. (1952) and Salk (1953) strongly supported the idea and suggested that the oil and the emulsifier were as important in attracting cells to the antigen site as in retaining the antigen there.

Quite a lot of evidence for the theory has been accumulated. Oakley et al. (1949) found that there was as much antibody in extracts from the site of injection of alum-precipitated diphtheria toxoid in the rabbit, as there was in extracts from the local lymph nodes. Askonas and Humphrey (1955) showed that the granuloma tissue at the site of injection of Freund's complete adjuvant in the rabbit was actively producing antibody and they calculated that it could account for 80% of the total antibody in the serum. White, Coons and Connolly (1955a & b) using fluorescent antibody techniques, showed

that there were many antibody-containing cells in the alum granuloma, but in contrast to Askonas and Humphrey's findings they were not seen when oil and tubercle bacillary wax was used as the adjuvant.

White, Coons and Connolly's (1955b) findings of "a remarkable systematized stimulation of the reticulo-endothelial system" with the oil adjuvant rather than local activity is difficult to reconcile with Askonas and Humphrey's showing that there was great antibody forming activity at the depot. The antibody and antibody containing cells found there may perhaps be considered as having been chemotactically attracted because of the presence of antigen. On the other hand, it may be that the actual placing of the depot, in panniculus muscle, loose connective tissue, fat layer, semi-intradermally, etc., may have some bearing on these differences.

Many other suggestions to account for the action of the depot-forming adjuvants have been made, either to stand alone, or as additions to the classical slow release theory. Landsteiner (1946) suggested that the particulate state of an alum precipitated antigen was probably important as it facilitated phagocytosis from the depot. Burnet (1959) suggested that a sensitive cell

able to react with the antigen at once and form a clone of antibody producing cells might not be present in the body at the time of vaccination. The slow release of antigen from the depot would then ensure that it was available for an extended period during which such a cell might arise by random mutation. However, there seems to be no evidence for the widely different response time between individuals of the same species which would be implied by this idea. The first appearance of detectable antibodies may show considerable variation in time, but the slow starters usually only show low peak titres implying a variation in quantity rather than in the time of initial stimulation.

Dale (1960, 1961) found that marked lymphatic proliferation occurred in the neighbourhood of Freund's adjuvant depots. This she considered might lead to a more efficient direction of the antigen to the local lymph nodes rather than its rapid dispersion into the blood. Some support was given to this suggestion by the work of Bishop (1961) who injected the antigen uncombined with, and at a different site from, oil adjuvant. He found that the latter was only effective if the lymphatic drainage from the two injection sites was the same.

The inflammatory response was found to be important by Wilner *et al*. (1963) who showed that there was a positive correlation between the effectiveness of various hydrocarbon oils as adjuvants and their irritant properties. Wilner *et al*. and Berlin (1960) also showed that the more viscous the emulsion, the less the adjuvant effect. This suggests that release of particles of emulsion from the main depot is important.

Oil alone has no adjuvant action according to Freund *et al*. (1948) and simple lipids such as ethyl palmitate, cholesterol oleate and glycerol trioleate seem either to have no effect at all when given with a particulate antigen or only to depress the antibody response. They are, however, very active in modifying the phagocytic activity of the reticulo-endothelial system (Stuart and Davidson, 1964).

On the other hand, Stanley (1950) showed that the lipoids of *Aspergillus fumigatus*, and *Listeria monocytogenes*, and also lecithin, augmented antibody production against *Salmonella typhi-murium*. Freund *et al*. (1948) found that lipids extracted from *M. tuberculosis* had an adjuvant effect, though not all of the tubercle wax D fractions tested by White *et al*. (1958) were active. Pound (1958) carried out experi-

ments which showed that these lipids acted only by stimulating the lymphoid and reticulo-endothelial tissues. They neither bound the antigen at the site of inoculation to make a depot, nor formed a more potent antigen by combining with it.

Dresser (1961) thought that lipid and lipidophilic substances showing adjuvant activity might in some way stimulate cells to take up antigen, possibly by damaging their cell membrances. The lipid A portion of the endotoxins of gram-negative bacteria is thought to be responsible for their adjuvant activity (Farthing, 1961). Thus it is possible that the oils do in fact have some subtle action on the antibody forming, or antigen recognizing tissues.

The most recent, detailed investigations, are those of McKinney and Davenport (1961) who used water-in-oil emulsion vaccines, and a model system of cotton wool pellets implanted subcutaneously. They concluded that an initial large loss of antigen from the injection site was important, that a continual leak of antigen from the depot was essential for the maintenance of titres at a high level, and that the early inflammatory response round the depot occurred during a critical period so that antigen was being released whilst cells were available

locally to react with it. By transferring the granuloma enclosed antigen depots to fresh mice they demonstrated that these were not antibody forming organelles of any importance.

All this evidence is difficult to sum up, isolated investigations have been carried out on such a variety of substances and mixtures of substances under different conditions that it is not surprising that many results are contradictory. With Freund's adjuvant in particular, it may be that it is acting both as a depot type adjuvant and also as one having more far-reaching effects when mycobacteria are added to make the 'complete' form. From the glimpses of their mechanism that we have gained so far, it appears that: the slow release of antigen over an extended period is important, that small dispersed depots may be as important as the large one at the injection site, that the concept of gross antibody formation round the main depot only applies in special cases, and that the inflammation and reaction set up by the adjuvant around the depots may lead to a better utilization of the antigen than occurs without adjuvant. That these depot-forming type of adjuvants have any more direct physiological action on the antibody forming cells is problematical.

## D. THE PRESENT INVESTIGATION

The objects of the present investigation were, first, to fill in some of the gaps in our knowledge of the basic pattern of antibody response to an antigen given in water-in-mineral oil emulsion, and second, to carry out experiments to help clarify the unresolved problems of their mode of action. Aspects of the basic response were chosen for study with reference to the practical use of adjuvant vaccines in man and animals; particularly with regard to the effect of subsequent injections of the same or other antigens and the formulation of adjuvants more easily handled than the usual viscous emulsions.

To simplify these investigations, the response of mice to a single subcutaneous dose of a pure protein antigen in water-in-oil emulsion was taken as the standard with which the other results could be compared.

A pure protein antigen was chosen to avoid the possible presence of a toxic or adjuvant moiety as might occur in an antigen derived from a micro-organism. This antigen was ovalbumin. It was prepared by chromatography because commercial, crystallized samples were found to contain conalbumin, an impurity reported to

interfere with the tanned cell agglutination test. Some further investigations on this point were carried out.

The antigen was given subcutaneously as this is the usual route for vaccination. Multiple doses were not used, for though they may raise very high titres, the presence of a secondary or even hyperimmune response is confusing. The prime requirement of an adjuvant should also be that an excellent response follows a single dose.

Mice were used as the experimental animals. Individuals are easily bled repeatedly and they could be obtained in the numbers necessary to carry out a wide range of experiments.

Mineral oils and emulsifiers are poorly defined materials. Toxicity tested batches supplied for the preparation of human vaccines were used to prepare the emulsions, in order to reduce this variable as much as possible.

Serum antibodies were titrated by the tanned cell agglutination test, a technique which is very sensitive, yet easy to carry out. After some investigation of variables in the method of preparing the cells, formalinized cells were coated in large batches and preserved by freezing. These cells could be used at any time and batches checked against each other for

variations in sensitivity.

In addition to studies of the mode of action of adjuvants in experimental animals, the author participated in trials with human volunteers. This was undertaken to study the antibody response and reactions to an adjuvant type influenza virus vaccine which was to be made available commercially in this country. The water-in-oil type of adjuvant was thus seen in action under field conditions.

# Part Two

# MATERIALS AND METHODS

## A. EXPLANATION OF SPECIAL TERMS AND ABBREVIATIONS USED IN THIS THESIS

Oil/Arlacel. This term indicates the standard mixture of nine parts of Drakeol 6-VR with one part of Arlacel A, sterilized by Seitz filtration.

Ovalbumin. Ovalbumin purified by ion exchange chromatography is meant when the term 'ovalbumin' without any qualification is used in descriptions of experiments carried out by the author.
Weights of ovalbumin given refer to the freeze dried material.

Saline. This is a 0.85 per cent W/V solution of sodium chloride in distilled water.

Titre. Titres are recorded as the reciprocals of the initial serum dilutions.

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Abbreviations.

CA = conalbumin.

cm = centimetre.

CM-cellulose = carboxymethyl-cellulose.

C.P.S. = cycles per second.

G = relative centrifugal force x gravity.

g = gramme.

G.M. titre = geometric mean titre.

H.A. unit = haemagglutinating unit.

IgG type antibody = 7S$\gamma$, $\gamma_2$, or $\gamma_{ss}$ antibodies.

IgM type antibody = $\gamma_1$M, $\beta_2$M, or 19S$\gamma$ antibodies.

m/a = milliamperes.

mgm or mg = milligramme.

ml = millilitre.

mm = millimetre.

M.S.E. = Measuring and Scientific Equipment Ltd., London S.W.1.

m$\mu$ = millimicron.

N.R.S. = normal rabbit serum.

OA = ovalbumin.

oz = ounce.

P = probability.

P.B.S. = phosphate buffered saline.

U.V. = ultra violet.

V = volts.

W/V = weight in volume.

$\mu$gm or $\mu$g = microgramme.

$\mu$l = microlitre.

## B. MATERIALS

Details of the materials used and their suppliers have usually been given with the first mention of each in the Methods or Results sections. Other materials and equipment are listed below.

Acetate buffer for ion-exchange chromatography

Solution A. 0.1 M ammonium acetate
(7.7 g $CH_3COONH_3$ in 1000 ml distilled water).

Solution B. 0.1 M acetic acid
(5.7 ml glacial acetic acid in 1000 ml distilled water).

Some of the combinations needed to obtain a desired pH are:

| pH | Solution A | Solution B |
|---|---|---|
| 4.2 | 14 ml | 34 ml |
| 4.4 | 19 ml | 31 ml |
| 4.7 | 27 ml | 23 ml |
| 5.0 | 35 ml | 15 ml |
| 5.3 | 43 ml | 7 ml |

Higher pH values were obtained by adding a 0.1 M solution of sodium carbonate as required.

Adjuvants

Freund's Incomplete Adjuvant (a mixture of

the mineral oil Bayol F, 8.5 parts, with Arlacel A, 1.5 parts) and Freund's Complete Adjuvant (as above but with the addition of 5 mg of dried, heat killed Mycobacterium butyricum to each 10 ml) were obtained from: Baird and Tatlock (London) Ltd., Chadwell Heath, Essex, the British agents of the Difco Laboratories Inc., Detroit, U.S.A.

Agglutination plates

World Health Organization plastic agglutination plates (8 x 10 wells) were obtained from: Prestware Ltd., Raynes Park, London S.W.20.

Aluminium hydroxide gel

A sample of 'Alhydrogel' brand of aluminium hydroxide gel was kindly supplied free by the manufacturers: Dansk Svovsyre-OG Superphosphat-Fabrik, Copenhagen.

Animals

The mice used in the experiments were either The Laboratory Animals Centre Grey strain, bred in the Bacteriology Department animal house; or Swiss white mice obtained from the M.R.C. Clinical Endocrinology Research Unit in Edinburgh. Both strains were random bred.

Rabbits, of various breeds, were obtained from the University of Edinburgh Small Animal Breeding Station, Easterbush.

'Aquaphor'

A sample of the ointment base 'Aquaphor' was presented to the laboratory by Professor S. Raffel of Stanford University.

'Arlacel'

A supply of Arlacel A was most kindly given by Dr. D. Hobson of Evans Medical Ltd. Arlacel A (mannide mono-oleate) is a product of the Atlas Powder Company, Wilmington, Delaware.

Carboxymethyl-cellulose

Whatman CM 70 carboxymethyl-cellulose powder was used for ion-exchange chromatography.

'Courlose' commercial CM-cellulose purchased from J.M. Steel and Co., Glasgow, was used for removing excess water from proteins held in dialysis tubing.

Conalbumin

A specimen of electrophoretically pure conalbumin was most kindly supplied by Dr. I.E. Lush of the Agricultural Research Council Poultry Research Centre, Edinburgh.

Corks

Small natural corks (3/8th x 1/4Fl x 3/16th inch) suitable for stoppering Durham tubes were obtained from Symington and Dumbreck, 32 South Portland Street, Glasgow C.5.

Crystalline ovalbumin

Twice re-crystallized ovalbumin was obtained from L. Light and Co. Ltd., Colnbrook, England.

Dialysis tubing

Visking cellulose tubing, size 18/32 or 32/32 was used. This is manufactured by the Union Carbide Corpn., U.S.A.

Distilled water

De-ionized, or occasionally glass distilled, water was used.

Durham tubes

Very small glass test tubes were used for collecting mouse blood, storing sera and for many other purposes. These were the Durham tubes normally employed to test for gas production by bacteria in sugar solutions. The most useful type were those supplied by G.W.S., sized 20/21 x 6/7 mm. Natural corks of a standard size to fit these tubes were obtained from Symington and Dumbreck, Glasgow. The tubes were supported during use in wooden blocks drilled with 9/32 inch holes.

After use, the Durham tubes were cleaned by putting them into a conical flask partly filled with a solution of detergent. The flask and

contents were gently agitated, as in a 'Bendix' washing machine, by being placed in a wire test tube basket attached to a 'Matburn' blood cell suspension mixer. When clean, the tubes were rinsed many times with tap water and distilled water before being dried in the hot air oven. On occasion it was also necessary to steep the tubes overnight in dilute caustic soda to remove traces of blood clot, etc.

'Matburn' blood cell suspension mixer

This most useful piece of equipment consists of a metal gramophone turntable inclined at an angle of about 45°, which can be made to revolve at a slow speed. The edge of the turntable has spring clips of various sizes to hold bottles and it was also found possible to attach a 6 x 6 x 6 inch wire test-tube basket to the centre. Larger bottles and flasks could be placed in this. Depending on the position in which a bottle was placed, it received very gentle or more vigorous agitation, but without the more violent type of shaking produced by machines of the reciprocating type. The mixer is supplied by Baird and Tatlock (London) Ltd.

Mineral oil

A bottle of Drakeol 6-VR was most kindly

supplied as a gift by the Pennsylvania Refining Company, Butler, Pennsylvania.

Miniature pipettes

Small Pasteur pipettes only 7 cm long were used extensively for the work reported in this thesis. They were prepared from soda glass tubing having an outside diameter of about 5 mm and an internal diameter of about 3 mm. Rods 11 cm long were cut from this material and drawn out in a small Bunsen flame to make two pipettes. These were cut to a length of exactly 7 cm each and then sorted into classes according to the diameter of their tips. The sorting was done with the assistance of a special gauge. This was a small sheet of copper with a V-shaped slot cut into it and marked off in four sections. After manufacture or washing the pipette tip sizes could rapidly be checked with this.

Size A. Those suitable for removing sera from blood samples, etc. These were the smallest and had a tip diameter of less than 0.5 mm.

Size B. Those used for bleeding mice from the eye. External tip diameter of about 0.7 mm.

Size C. Those used for preparing 1:10 dilutions of sera for the tanned cell

agglutination test. Tip diameter about 1.5 mm.

Size D. Those used for making doubling dilutions with the aid of an automatic syringe. They were slipped into a hole bored through a cork attached to the tip of the syringe. These pipettes were the widest with a tip diameter of over 1.75 mm.

Immediately after use all pipettes were dropped point first into a 250 ml plastic beaker filled with a detergent solution. After soaking in this for some time they were washed well with many changes of tap-water followed by changes of distilled water before being dried in the oven. Being all of the same length they were very easy to handle in the beakers.

Occasionally the pipettes were allowed to soak in dilute caustic soda for a few hours or overnight. This was particularly necessary to clean those which had been used with whole blood.

Tween

Tween 80 (T.B. Culture Grade) was obtained from Honeywill and Stein Ltd., London. Tween 80 is polyethylene sorbitan mono-oleate.

Ultrasonic disintegrator

An M.S.E. (Measuring and Scientific Equipment Ltd., London S.W.1)/Mullard, ultrasonic disintegrator was used to form emulsions. The titanium probe with a diameter of 1 cm was found to be the most suitable. The frequency of the ultrasound produced could be varied from 18,000 to 20,000 c.p.s. The machine was tuned within this range to produce maximum agitation in the vial of emulsion.

## C. METHODS

## 1. The separation of egg-white proteins by ion-exchange chromatography on carboxymethyl-cellulose

Ion exchange, the reversible exchange of ions in a solution with those of an insoluble material, is well known in its classical form in the domestic water softener. The development of synthetic resins led to its much wider application particularly with organic materials. Peterson and Sober (1956) first employed carboxy-methyl-cellulose for the separation of proteins by this technique and Rhodes, Azari and Feeney (1958) used the same material to separate the proteins found in egg-white.

Rhodes et al. applied the whole egg-white at a low pH to a column packed with carboxy-methyl-cellulose. By passing a series of buffers of increasing pH through the column the component proteins of the egg-white were separately eluted. Their method was followed in the present work.

### a) Pre-treatment of the carboxymethyl-cellulose (CM-cellulose)

Twenty-five grams of CM-cellulose were steeped overnight in distilled water. The swollen material was then placed in a large conical flask with side arm and de-aerated under the vacuum from a filter pump. This took

several hours with magnetic stirring and frequent shaking.

The supernatant fines were next removed from the slurry. The mixture was allowed to settle for some minutes until a clear cut surface could be seen above the deposit. The supernate and small particles still suspended were then removed by sucking them off with a pipette attached to the filter pump. This was repeated several times until the heavier material would settle out in about five minutes leaving a substantially clear supernate.

Unless the removal of fines was done adequately it was found that the columns gave a very poor flow rate.

b) Column packing

A column of the form shown in figure 1 was used. This was the receiver of the Parnas-Wagner automatic discharge arrangement of a micro Kjeldahl apparatus (part number NR 653; Gallenkamp and Co. Ltd.). It was 20 cm long and 5 cm in diameter.

The narrow portion at the bottom was filled with glass beads and these were covered with a thin layer of glass wool to obtain a flat base for the packing.

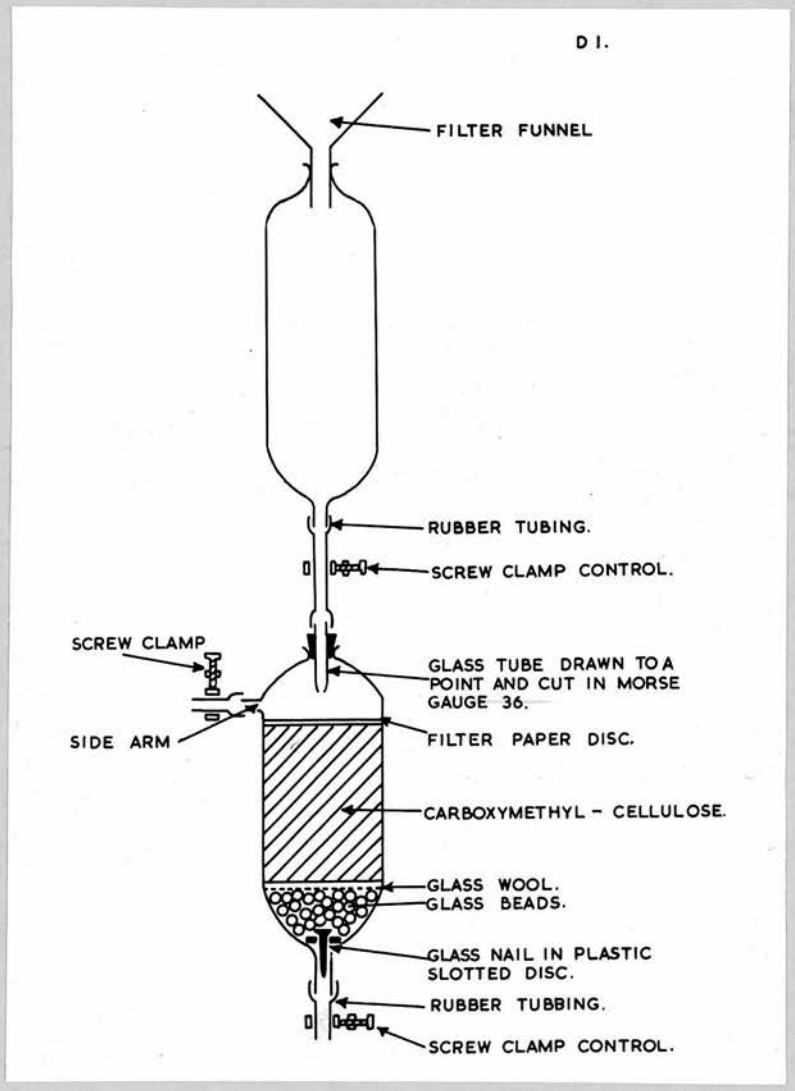



Fig.1. Separation of egg-white proteins by ion exchange chromatography on CM-cellulose. Details of the column packing and arrangement of the apparatus for elution by stepwise pH changes.

About one fifth of the carboxymethyl-cellulose, which had been soaked and de-aerated as detailed above, was fed into the column as a slurry in distilled water. The column had already been filled with water to the level of the glass wool and as the CM-cellulose settled, water was allowed to escape slowly by opening the screw clamp at the bottom. When the surface of the CM-cellulose was still just wet, a wide glass rod was used to tamp down the packing as tightly as possible. More water was allowed to escape as this was done, care being taken to avoid any entrapment of air. More aliquots of the slurry were added and tamped down as hard as possible until the column had been filled as shown.

The packing occupied a volume of about 150 ml and it was found that when really well packed the column held water by capillary action. Even if allowed to run dry, air was not drawn into it.

A disc of filter paper was placed over the surface of the CM-cellulose to prevent its disturbance by the eluting and washing buffers dropped on to it. Air was passed into the glass bead packing at the bottom to increase the crispness of the changes in the components eluted.

The columns were kept at 4° when not in use, without any preservative.

c) Cleaning the column before use

Several litres of N/10 hydrochloric acid and N/10 sodium hydroxide were alternately and quickly passed through the column. Finally one litre of 0.1M (7.7 g/litre) ammonium acetate was passed through it. The column was then ready to be equilibrated with the starting buffer.

This equilibration was done at a very slow flow rate and samples were taken at intervals to be checked for pH. When several hundred millilitres of buffer at the wanted pH had passed through, the column was considered to be ready for use.

All buffers, etc., were de-aerated and filtered before being passed through the column.

d) Preparation of the materials to be fractionated

Fresh eggs were broken into a large Petri dish and the thin portion of the white carefully transferred to another container with a Pasteur pipette. This egg-white was then homogenised for 5-10 minutes in an M.S.E. (Measuring and Scientific Equipment Ltd.) homogeniser at a speed just below that at which marked frothing occurred. It was then put into Visking dialysis tubing and

dialysed against several changes of 0.1M acetate buffer, pH 4.2, for two days at 4°. During this time a precipitate of ovomucin developed. This precipitate was removed from the egg-white by centrifugation at 3000 G for 30 minutes in the cold.

Partly purified egg-white proteins in solution in distilled water were dialysed overnight against the starting buffer to bring them into equilibrium with it. If any precipitate or denatured ovalbumin was present the solution was filtered before being applied to the column.

e) Application of egg-white proteins to the column

To avoid contamination of the upper flask (fig. 1), the stopper at the top of the column itself was removed and the dialysed material poured directly on to the CM-cellulose. This was done with care and in small volumes at a time to avoid frothing. The screw clamp at the bottom of the column was opened to allow the displaced liquid to run out and it was closed again when all the liquid had been absorbed into the packing.

f) Elution of proteins from the column

(i) Using stepwise pH changes

After the egg-white protein had been

applied, a small quantity of the starting buffer was allowed to run in to form a shallow pool on top of the CM-cellulose. The eluting buffers were then run through, in order, from the reservoir above as shown in figure 1. The rate of flow was controlled with a screw clamp. It was found convenient to draw off to a point, the glass tube leading into the column. It was then cut, as one would cut a dropping pipette, to Morse gauge 36. A dropping rate of 76 per minute then gave a flow of about 4 ml per minute. This was the usual elution rate used with this column.

All of the previous buffer, including that forming the shallow pool above the packing, was allowed to enter the column material before the next buffer was applied.

(ii) Using a gradient buffer

This is a method by which a buffer of continuously varying pH can be passed through the column.

The apparatus was arranged as shown in figure 2. The starting buffer (300 ml) was placed in the container B and stirred magnetically. Buffers of increasing pH were added from the reservoir A, the flow being controlled by the screw clamp. The mixed buffer siphoned out of

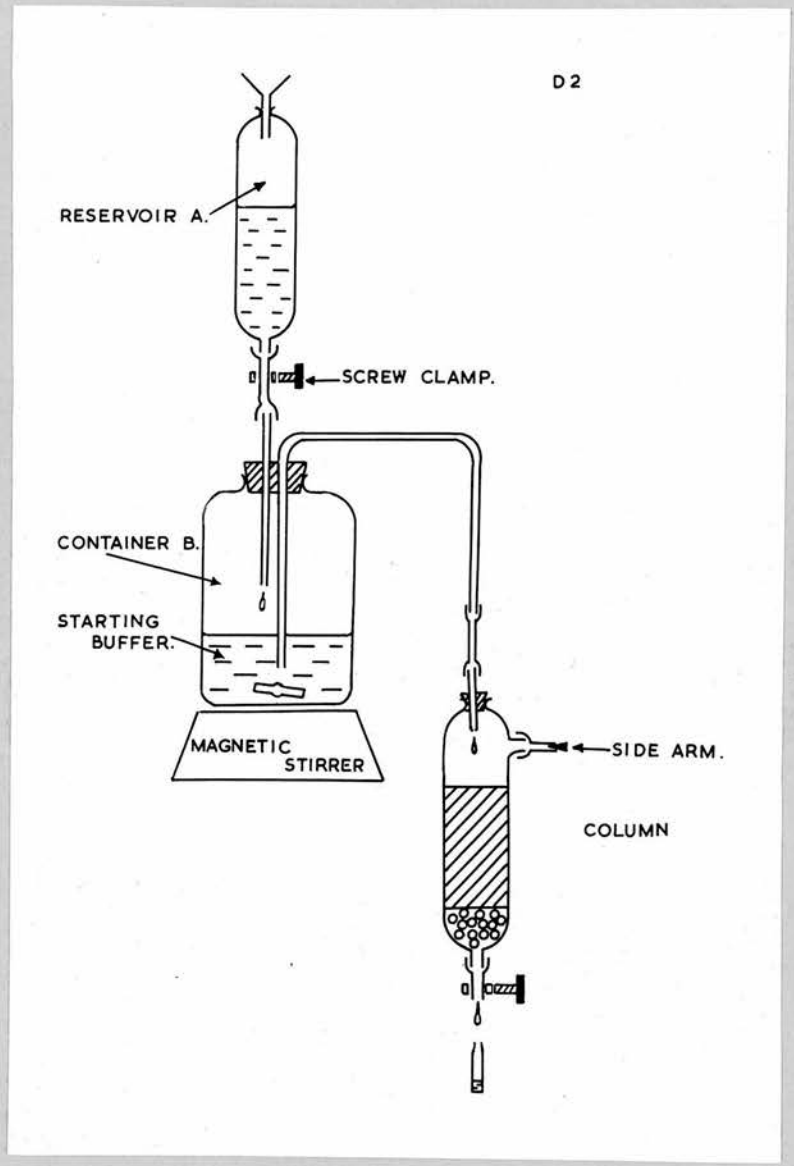



Fig.2. Chromatography of egg-white proteins on CM-cellulose. The apparatus used for elution of the proteins by a gradient pH change. Details of the column packing are shown in fig.1.

container B and passed through the column.

The buffer was allowed to fall into a shallow pool above the packed surface of the CM-cellulose. This pool was introduced at the start by opening the side arm for a short while. As the whole system was airtight the flow rate could be accurately controlled by the screw clamp below reservoir A. A flow rate of 76 drops per minute was used.

g) Fraction collection

With both methods, fractions of approximately 20 ml each were collected by hand in 1 oz screw capped vials.

h) Treatment of the column after use

After use the columns were purged of remaining proteins and prepared for the next fractionation by passing through them the following solutions:

500 ml N/10 NaOH

500 ml N/10 HCl

500 ml N/10 NaOH

750 ml 0.1M ammonium acetate

and then about 1000 ml of the starting buffer until the eluate was of the same pH.

If bands of colour were seen passing down the column whilst the second wash of NaOH was

being passed, the washing with acid and alkali was repeated.

i) Measurement of pH

An Electronic Instruments Ltd. Model 23A Direct Reading pH meter with ordinary glass and calomel electrodes was used to measure the pH of the buffers used and of the fractions collected. When great accuracy was required the special 'extended range facility', which doubles the scale over a smaller range, was used.

The calibration of the instrument was checked at frequent intervals (before each buffer and between every five or six fractions) with a standard buffer prepared from tablets (Burroughs Wellcome and Co.).

j) Measurement of protein concentration

(i) The biuret method

This was used when fractions contained a large quantity of protein such as those from the initial fractionation of egg-white. The method used was adapted from that of Fleury and Eberhard (1951).

Reagent. The following solution was made up:

| | | |
|---|---|---|
| $Cu_2SO_4 \cdot 5H_2O$ | 0.75 | g |
| Potassium sodium tartrate | 3.0 | g |
| Distilled water | 150 | ml |

When the solids had dissolved 350 ml of 0.1 N NaOH was added. The reagent was used on the day of preparation.

Method. One ml of each fraction was added to 4 ml of the reagent and the mixture allowed to stand for half an hour. The light absorption of the liquid was then measured with an EEL portable colorimeter Model A (Evans Electro-selenium Ltd.). Buffer and biuret reagent were used as a blank. The green filter OGR 1 was found to be the most suitable.

The readings of the colorimeter were used as they stood. No attempt was made to obtain absolute values by comparison with protein solutions of known strength.

(ii) Spectrophotometric method

When there was little protein present it was determined by measuring the optical density of the solution in ultra-violet light at 280 mμ. A Unicam spectrophotometer type SP 500 was used. The light source was a hydrogen arc and fused silica cells were used for the samples. If necessary the samples were suitably diluted to obtain a reliable reading.

Protein concentration was recorded as

optical density at 280 mμ and not converted to protein.

A great advantage of the method was that no portion of each fraction was lost in making the estimation.

k) Preservation of the purified proteins

Fractions containing the wanted materials were pooled and filled into dialysis tubing. A layer of commercial grade carboxymethyl-cellulose powder ('Courlose', J.M. Steel and Co., Glasgow) was placed in an aluminium bread tin, the filled dialysis tubes placed on top and more of the CM-cellulose added to cover them. The tin was stored at 4° until the desired reduction in the volume of protein solution had taken place.

The now jelly-like CM-cellulose adherent to the tubes was washed off with cold tap water and the tubes tied off short to confine the protein to a small volume. To remove the remaining buffer salts the tubes were dialysed for several days at 4° against changes of distilled water.

Finally, the materials were freeze dried in an Edwards High Vacuum Ltd. Speedivic Model 10P freeze dryer. This type of machine can deal readily with large quantities of liquid in flasks. Primary drying only was carried out, without the

use of phosphorus pentoxide.

The resultant powder was stored in glass vials closed with screw caps and rubber washers.

2. Short method for preparing recrystallized ovalbumin from egg-white by salting out with ammonium sulphate

This was adapted from the methods of Sørensen (1915) and Cole (1933, as described by Koch and Hanke, 1953). Fresh egg-white is acidified with acetic acid which causes some precipitation of ovomucoid. It is then brought to 44 per cent saturation with ammonium sulphate which completes the precipitation of the ovomucoid and of some of the globulin. After removal of the precipitate, the solution is brought to 52 per cent saturation with ammonium sulphate and the ovalbumin crystallizes out. This ovalbumin is removed, dissolved in distilled water and recrystallized by once again bringing the saturation of the solution with ammonium sulphate to 52 per cent.

All the processes were carried out at room temperature, centrifugations being carried out in a refrigerated centrifuge controlled at that temperature.

Detailed method

a) A stock saturated solution of ammonium sulphate was prepared by dissolving:

550 g ammonium sulphate

6.8 g sodium acetate 3 $H_2O$

and 3 ml glacial acetic acid

in 750 ml of warm distilled water. The solution was then filtered and checked to see that crystals separated on cooling. If necessary a little more ammonium sulphate was added.

b) The whites of six medium-sized fresh eggs were collected, care being taken to avoid contamination with yolk. This egg-white was gently homogenised in a M.S.E. homogeniser, frothing being avoided as far as possible.

c) To 100 ml of the homogenised material 11 ml of N acetic acid was added (5.7 ml glacial acetic acid in 100 ml distilled water).

After stirring for one minute, 22 g of solid ammonium sulphate was added to the mixture. Stirring was continued for 10 minutes until it had completely dissolved.

d) To the resultant solution, 22 ml of the stock saturated solution of ammonium sulphate was added over a period of 5 minutes whilst stirring was continued.

e) The resultant precipitate was spun down by

centrifugation at 3000 G for 30 minutes. The supernate was removed and filtered.

f) Whilst the filtration was being carried out, the precipitate remaining in the centrifuge bottles was washed with 44 per cent saturated ammonium sulphate solution. It was then re-centrifuged at 3000 G for 15 minutes and the supernate added to that being filtered in step e) above. The precipitate was then discarded.

g) Whilst gently swirling the filtrate from step e) in a conical flask, the following additions of ammonium sulphate solution were made:

26 ml of 55 per cent saturated solution in one lot

and 52 ml of 55 per cent saturated solution in small amounts over 30 minutes.

A precipitate appeared which, after being allowed to stand for several hours or overnight, showed the typical 'shot silk' swirls of ovalbumin crystals when disturbed. These were checked under the microscope.

h) The precipitate was resuspended and the following additions of ammonium sulphate made:

20 ml of 55 per cent saturated solution over 20 minutes

and 48 ml of 70 per cent saturated solution over 20 minutes.

The mixture was then allowed to stand for a few hours or overnight.

i) The precipitate which had formed (ovalbumin) was then spun down by centrifugation at 1500 G for 30 minutes, the supernate discarded and the pellet washed once with 51 per cent saturated ammonium sulphate.

The ovalbumin was further purified by re-crystallization.

j) The pellet was carefully dissolved in 30 ml of distilled water, and 40 ml of 70 per cent saturated ammonium sulphate solution added slowly. The mixture was continually stirred during this addition so that any precipitate produced dissolved up again.

k) The solution was filtered, and the paper washed through with 20 ml of 40 per cent saturated ammonium sulphate. To the filtrate was added 120 ml of 55 per cent saturated ammonium sulphate solution over a period of 30 minutes. A precipitate appeared.

l) After standing for one hour, 40 ml of 70 per cent saturated ammonium sulphate solution was added and the mixture allowed to stand for a few hours or overnight.

m) The recrystallized ovalbumin was removed by centrifugation at 1500 G for 30 minutes, the

supernate discarded and the pellet washed with 51 per cent saturated ammonium sulphate solution.

n) The ammonium sulphate was removed from the recrystallized ovalbumin by dissolving it in tap water and then dialysing it against running tap water overnight.

Note. It was occasionally found that precipitates did not appear as expected in steps g) and k). This appeared to be due to the great day-to-day variations in the room temperature of the Edinburgh laboratory affecting the saturation point of the solutions. Small quantities of the stock saturated solution of ammonium sulphate were therefore added to the preparation until a precipitate was seen.

### 3. Routine method for the further purification of recrystallized ovalbumin by chromatography on carboxymethyl-cellulose

Ovalbumin, partially purified by crystallization, was applied to a carboxymethyl-cellulose column. A very pure ovalbumin preparation was eluted by a single pH change leaving any other unwanted materials on the column. Elution could be followed visually and checked by measuring the pH of the eluate. Details of the method used are as follows:

A column was packed with CM-cellulose and cleaned and equilibrated to pH 4.2 as detailed earlier. The apparatus was arranged as shown in figure 1.

After dialysis against pH 4.2 acetate buffer overnight, once recrystallized ovalbumin was applied to the column by the method previously described. The solution was passed through a filter paper to remove any insoluble particles.

After application, a white band could be seen in the upper third of the column. This represented the ovalbumin. It was more opaque than the rest of the packing and changes in its position during elution could best be seen by transmitted light from an Anglepoise lamp suitably masked with cardboard.

The column was washed with 250 ml of buffer at pH 4.4 to remove any ovomucoid, etc.; this did not affect the position of the white ovalbumin band.

Ovalbumins A1 and A2 were eluted by passing 300 ml of buffer at pH 4.65-4.70 through the column. The movement of the white band was watched and collection of the eluate started as soon as it neared the bottom of the packing. Collection was continued until all of this buffer had passed through, making a total of about

150 ml. Alternatively, the eluate was collected in 50 ml fractions, and all those showing a pH of 4.45 or above were assumed to contain ovalbumin. A sample was set up in double diffusion against an anti-egg-white serum to confirm its purity.

The eluate containing ovalbumin was reduced in volume with commercial grade CM-cellulose. Any remaining buffer was removed by dialysis against changes of distilled water and the material was then freeze dried.

4. Characterization of the egg-white proteins

The proteins in egg-white and purified preparations of them were characterized by the three techniques of electrophoresis, immuno-electrophoresis, and immunodiffusion.

Proteins will move in an electric field (electrophoresis), the extent of their travel being determined by the charge on them and the nature of the material in which they are supported. This effects a separation of the individual proteins.

If the electrophoresis is carried out in a solid support such as paper or agar, the positions of the proteins can be revealed by specific staining. Another method is to apply

an antiserum which will reveal its corresponding protein antigens by precipitating with them. This latter method, called immunoelectrophoresis, is usually more sensitive as it combines the advantages of electrophoresis with those of immunodiffusion.

In immunodiffusion an antigen and antibody are allowed to diffuse towards each other in a support such as agar. Precipitation occurs along a line where they meet and are present in equivalent proportions. When several antibody-antigen systems are present at the same time, the precipitation lines for each are often separated. This is because the relative concentrations of the materials, their abilities to diffuse through the support, and thus the position at which equivalence will occur, will differ for each system.

a) Electrophoresis in cellulose acetate paper

This was carried out in a Shandon electrophoresis box in conjunction with a Vokam constant current, constant voltage, power supply (Shandon Scientific Co. Ltd., London). Strips of 'Oxoid' cellulose acetate electrophoresis paper, 10 x 5 cm, were soaked in veronal buffer pH 8.6 and ionic strength 0.1, and mounted in the box.

Connection to the two buffer compartments was made with filter paper strips. The compartments had been filled with the same buffer, prepared from ready mixed salts supplied by L.K.B. Produkter A.B., Sweden.

The samples were applied to a marked position at the cathode end of the strips with a capillary tube. Current at 4 m/a per strip and a starting voltage of 200 V was allowed to run for a period of 2-4 hours with the power pack at the constant current setting. Any variations in this procedure are noted in the text.

After electrophoresis, the strips were dried in an oven at 100° for 10 minutes and then stained with either Ponceau S or with Nigrosine as follows:

Ponceau S (G.T. Gurr Ltd., London) was used as a 0.15 per cent solution in 3 per cent trichloracetic acid. The strips were soaked in this for five minutes, cleared in 5 per cent acetic acid, washed well in distilled water and dried under pressure between sheets of blotting paper.

Nigrosine (G.T. Gurr Ltd., London) was used as a 0.0025 per cent solution in 2 per cent glacial acetic acid in distilled water. The electrophoresis strips were stained with this

for 1-4 hours or overnight and the excess stain washed out with many changes of tap water. They were then washed with distilled water and dried as above.

b) Immunoelectrophoresis

The micro-immunoelectrophoretic technique of Scheidegger (1955, as described by Crowle, 1961) was generally followed.

Clean standard microscope slides were coated with a layer of agar about 3 mm thick. This agar was made up as one per cent New Zealand or Ionagar No. 2 (Oxoid) in 0.02 M phosphate buffer at pH 7.2 (Cruickshank, 1960). Two holes with a slot between them were cut in the agar on the slide using 2 mm diameter cut-off needles and the two halves of a razor blade, all mounted in a cork. The centres of the holes were removed by suction with a Pasteur pipette but the centre trench was not opened until after electrophoresis.

The slides were supported on plastic pins in the same Shandon electrophoresis box as that used for electrophoresis on cellulose acetate paper. Connections were made to the buffer wells (containing 0.02 M phosphate buffer) by strips of blotting paper. It was found

necessary to use many layers of blotting paper to reduce the resistance as much as possible and therefore increase the effective voltage drop across the agar itself.

The specimens (usually 1 per cent solutions in phosphate buffer) were put into the wells and the current allowed to flow at 2.5 m/a per slide and at an initial voltage of 50 V. The constant current setting was used.

After two hours the slides were removed from the box, the agar removed from the centre trench and this then filled with serum. The slides were incubated in moist chambers at 37° overnight and sometimes for some days subsequently at 4° until the lines were sufficiently developed.

The slides were then washed, dried, and stained in the same manner as the immunodiffusion plates (see below). The figures in this thesis are direct enlargements made from them.

c) Immunodiffusion

The Ouchterlony method of double diffusion, as described by Kabat and Mayer (1961), was used.

Petri dishes were filled with a layer of agar about 3-4 mm deep. Wells were cut in it with a 7 mm diameter cork-borer or with a Feinberg cutter (Shandon Scientific Co., London).

The centres of these wells were sucked out with a wide-mouthed pipette attached to a filter pump.

The Petri dishes were clean but not otherwise specially prepared, and the bottoms of the wells were not sealed with agar. The agar was prepared as one per cent New Zealand agar or Ionagar No. 2 (Oxoid) in saline, phosphate buffered saline or tris buffer at pH 7.2 (Cruickshank, 1960).

The wells were filled with the reagents (antigens were made up as a 1 per cent solution in distilled water or saline and the sera were undiluted, unless otherwise stated) and the results were read after overnight incubation at 37° or after a further 24-48 hours in the refrigerator at 4°. The wells were never refilled or topped up whilst the reaction was taking place.

The plates were preserved by soaking for one day in normal saline and for two days in changes of distilled water. The agar was then removed from the Petri dish and dried on to a clean lantern plate cover glass. It was then stained with naphthaline black made up as a one per cent solution in a mixture of methylated spirit 50 parts, glacial acetic acid 10 parts, and distilled water 40 parts. After staining for 5-10 minutes the plates were cleared with

changes of the same solvent. They were dried at room temperature and in some cases varnished later with Griffin picture varnish (Windsor and Newton Ltd.).

The figures in the text are direct enlargements made from the stained plates.

5. Preparation of precipitating sera

Anti-sera against the antigens: whole egg-white, commercial twice recrystallized ovalbumin, and chromatographically purified ovalbumin were prepared in rabbits. Schedules of the inoculations given and of the designations of the sera obtained during the courses of immunization are given in tables 1, 2 and 3.

6. Capillary tube precipitation test

Doubling dilutions of antigen (usually a one per cent solution) were made with normal saline in the wells along one edge of a W.H.O. plastic agglutination plate.

Standard capillary tubes of 1.5 mm external diameter were marked with a grease pencil at points 4 and 8 cm from one end. Neat antiserum was allowed to fill a tube to the first mark and the tip was wiped clean with paper tissue.

Table 1. Schedule of inoculations given to a rabbit for the preparation of antisera against whole egg-white.

| Day | 0 | 18 | 30 | 58 | 69 | 145 | 155 |
|---|---|---|---|---|---|---|---|
| Antigen | EW dil. 1:5 | EW dil. 1:5 | - | EW dil. 1:5 | - | 'Globulin' | - |
| Amount injected | 1 ml | 1 ml | - | 0.5 ml | - | 10 mg | - |
| Vehicle | Complete adjuvant | Saline | - | Saline | - | Saline | - |
| Route | S/C | S/C | - | S/C | - | S/C | - |
| Serum sample designation | - | - | R5C | - | R5E | - | R5F |

EW = whole egg-white.

'Globulin' = Globulin peak from chromatography of whole egg-white (see figure 22).

Complete adjuvant = Freund's complete adjuvant (Difco).

S/C = subcutaneously.

Table 2. Schedule of inoculations given to a rabbit for the preparation of antisera against twice recrystallized ovalbumin and against whole egg-white.

| Day | 0 | 5 | 8 | 15 | 22 | 93 | 111 | 120 |
|---|---|---|---|---|---|---|---|---|
| Antigen | 2xCryst OA | 2xCryst OA | 2xCryst OA | 2xCryst OA | - | Chromat. OA | EW dil. 1:5 | - |
| Amount injected | 15 mg | 10 mg | 10 mg | 10 mg | - | 20 mg | 1 ml | - |
| Vehicle | Incomplete adjuvant | Saline | Saline | Saline | - | Saline | Saline | - |
| Route | S/C | S/C | S/C | S/C | - | S/C | S/C | - |
| Serum sample designation | - | - | - | - | BWC | - | - | BWE |

2 x Cryst OA = commercial twice recrystallized ovalbumin.

Chromat. OA = chromatographically purified ovalbumin batch C18 (figure 16)

S/C = subcutaneously.

Incomplete adjuvant = Freund's incomplete adjuvant (Difco).

EW = egg-white.

Table 3. Schedule of inoculations given to a rabbit for the preparation of antisera against ovalbumin purified by chromatography.

| Day | 0 | 16 | 44 | 56 | 83 | 94 |
|---|---|---|---|---|---|---|
| Antigen | OA (1) | - | OA (1) | - | OA (2) | - |
| Amount injected | 10 mg | - | 5 mg | - | 5 mg | - |
| Vehicle | Complete adjuvant | - | Saline | - | Saline | - |
| Route | S/C | - | S/C | - | S/C | - |
| Serum sample designation | - | R4B | - | R4C | - | R4E |

OA (1) = chromatographically purified ovalbumin batch C18 (figure 16).

OA (2) = chromatographically purified ovalbumin batch C21 (figure 16).

Complete adjuvant = Freund's complete adjuvant (Difco).

S/C = subcutaneously.

Antigen solution from one of the wells in the plastic plate was then allowed to fill the tube until the top edge of the liquid in it reached the upper mark. The tube was next tilted to bring the contents to the centre and, whilst holding a finger over one end, the other was thrust vertically into a piece of Plasticine mounted along a strip of wood. Tubes were prepared in this way with each antigen dilution and placed in order along the Plasticine.

When the set was complete, it was incubated at 37° for two hours and then left on the bench overnight. Next morning the length of precipitate in each tube was measured with the help of a strip of millimeter graph paper and a magnifying glass. Where all of the precipitate had not fallen to the bottom of the tube, the length of each portion was measured and added together.

7. Absorption of sera with ovalbumin or conalbumin prior to their titration with tanned cells coated with ovalbumin or with conalbumin or with a mixture of the two antigens (Part 4, Experiment 2)

Experiments were designed to determine whether conalbumin was the dominant partner when used with ovalbumin in the tanned red cell agglutination test. For these experiments sera

were required which had been absorbed to remove anti-ovalbumin or anti-conalbumin antibodies.

Precipitin curves with respect to ovalbumin and conalbumin for the sera R4B and R4C (anti-ovalbumin), BWE and R5C (anti-egg-white), and BWC (anti-commercial crystalline ovalbumin), were constructed. Tables and graphs for some of these are presented in tables 9 and 10 and figures 34 and 38. Tables 5 and 6 show the results for the remaining combinations of serum and antigen.

The absorptions were carried out by mixing neat serum with an equal volume of antigen diluted to a point just on the antigen excess side of equivalence, or a point taken to represent it.

The dilutions of one per cent antigen solution used are shown in table 4. After being mixed with the antigen the sera were incubated at 37° for 2 hours and then at 4° overnight. Any precipitates formed were then removed by centrifugation at 3000 G for 30 minutes at 4°.

Table 4. Dilutions of one per cent antigen solution used to absorb sera to be tested with cells coated with ovalbumin, or conalbumin, or with a mixture of them

| Antigen | Serum | | | | |
|---|---|---|---|---|---|
| | R4B | R4C | BWC | BWE | R5C |
| Conalbumin: | Neat | Neat | 1:8 | 1:16 | 1:2 |
| Ovalbumin: | 1:16 | 1:4 | 1:32 | 1:16 | 1:8 |

Table 5. Heights of precipitate formed in capillary tubes by the reaction of neat serum and an equal volume of a one per cent solution of ovalbumin (C18), diluted as shown.

| Serum | Dilution of antigen - 1 part in: | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 | |
| R4B (anti-OA) | 1.5 | 1.5 | 3.25 | 4.0 | 3.5 | 4.0 | ND | 2.25 | 2.0 | 1.5 | mm |
| BWC (anti-cryst OA) | 0.75 | 1.0 | 1.0 | 1.25 | ND | 1.5 | 1.75 | 1.5 | 1.25 | 1.0 | mm |
| BWE (anti-EW) | 1.0 | 1.75 | 2.25 | 2.25 | 3.0 | 2.0 | 2.25 | 2.0 | 1.75 | 0.25 | mm |

ND = not done. OA = ovalbumin.
cryst OA = commercial, twice recrystallized OA. EW = egg-white.



Table 6. Heights of precipitate formed in capillary tubes by the reaction of neat serum and an equal volume of a one per cent solution of conalbumin (D28), diluted as shown.

| Serum | Dilution of antigen - 1 part in: | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 | |
| R4B (anti-OA) | 1.0 | 0.25 | Tr | 0 | 0 | 0 | 0 | 0 | 0 | 0 | mm |
| BWC (anti-cryst OA) | 0 | 0.75 | 1.0 | 1.0 | 1.0 | 1.0 | 1.0 | 0.75 | 0.75 | 0.25 | mm |

Tr = trace. OA = ovalbumin.
cryst OA = commercial, twice recrystallized OA. 0 = no precipitate.

8. Antibody nitrogen determinations

Where sufficient serum was available, antibody nitrogen estimations were carried out on the sera used in the experiments to investigate the influence of conalbumin in the tanned cell agglutination test (Part 4, Experiment 2).

The equivalence points of the sera were determined by capillary tube precipitation tests. For the preparation of precipitates, 0.1 or 0.2 ml volumes of neat serum and a dilution of antigen just on the antigen excess side of equivalence, were mixed in Durham tubes. These were then corked and inverted several times to mix the contents. After incubation at 37° for one hour they were left overnight in the refrigerator at 4°.

In the morning the tubes were centrifuged at 3000 G for 30 minutes at 4°. The supernates were removed and some of them preserved for cross precipitation. The supernates which were not kept were checked by capillary tube precipitation to ensure that they did not contain unprecipitated antibody.

The precipitates were then washed twice with cold saline (4°). Their nitrogen content was measured by the Markham micro-Kjeldahl technique as described by Kabat and Mayer (1961). An

ordinary 2 ml burette was used for the titrations. It was found that 0.01 ml volumes could be added to the distillate if the burette was used with the tip touching the surface of the liquid instead of allowing drops to fall from it. The titration was done with N/100 $H_2SO_4$ and the indicator used was methylene blue/methyl red (Kabat and Mayer, 1961).

The first step in these estimations was to determine how much nitrogen the antigens themselves contained.

a) Nitrogen content of the antigens

The nitrogen content of chromatographically prepared ovalbumin (batch M) and a conalbumin containing material D10 were measured by the micro-Kjeldahl method. The results for 1 per cent solutions of both antigens are shown in table 7.

b) Antibody nitrogen in the sera

The specific precipitates produced by the interaction of various sera and the antigens were analysed by the micro-Kjeldahl technique as described above. In some cases, where the serum contained antibodies to more than one of the antigens, the supernate remaining after precipitation with the first antigen was

re-extracted with the second antigen. The results of the determinations are given in table 8.

Table 7. Nitrogen content of the antigen solutions.

| Dilution of solution | Volume analysed | Nitrogen content by the micro-Kjeldahl method (µg in sample) | Nitrogen content of the original solution (µg/ml) |
|---|---|---|---|
| a) 1% Ovalbumin | | | |
| 1:4 | 0.8 ml | 232 | 1160 |
| Neat | 0.2 ml | 227 | 1130 |
| Neat | 0.2 ml | 269 | 1340 |
| | | mean = | 1210 µg/ml |
| b) 1% Conalbumin | | | |
| 1:4 | 1.0 ml | 207 | 828 |
| Neat | 0.2 ml | 167 | 830 |
| Neat | 0.2 ml | 162 | 805 |
| | | mean = | 821 µg/ml |

9. Sterilization of materials and equipment

Antigen solutions and the oil/Arlacel mixture were sterilized by Seitz filtration through Ford's grade SB filter pads. This was either carried out with a conventional filter and water pump, or with a Hemming type miniature

Table 8. Antibody nitrogen content of sera.

| Serum | Previous absorption if any | Volume of serum analysed | Antigen used | Antigen dilution (of 1% solution) | Volume of antigen used | µg nitrogen in: Precipitate | µg nitrogen in: Antigen used | Antibody nitrogen in: ppt. (µg) | Antibody nitrogen in: 1 ml serum (µg) |
|---|---|---|---|---|---|---|---|---|---|
| Anti-egg-white sera | | | | | | | | | |
| R5C | - | 0.1 | OA | 1:64 | 0.1 | 25.2 | 1.8 | 23.4 | 234 |
| R5C | - | 0.1 | OA | 1:64 | 0.1 | 25.2 | 1.8 | 23.4 | 234 |
| R5C | - | 0.2 | OA | 1:64 | 0.2 | 50.4 | 3.6 | 46.8 | 234 |
| R5C | CA | 0.2 | OA | 1:64 | 0.2 | 50.4 | 3.6 | 46.8 | 234 |
| | | | | | | | | mean | 234 |
| R5C | - | 0.1 | CA | 1:32 | 0.1 | 8.4 | 2.5 | 5.9 | 59 |
| R5C | - | 0.2 | CA | 1:32 | 0.2 | 15.4 | 5.0 | 10.4 | 52 |
| R5C | - | 0.1 | CA | 1:32 | 0.1 | 5.6 | 2.5 | 3.1 | 31 |
| R5C | OA | 0.2 | CA | 1:32 | 0.2 | 15.4 | 5.0 | 10.4 | 52 |
| | | | | | | | | mean | 48 |
| BWE | - | 0.2 | OA | 1:64 | 0.2 | 11.2 | 3.6 | 7.6 | 38 |
| BWE | - | 0.1 | CA | 1:32 | 0.1 | 11.2 | 2.5 | 8.7 | 87 |
| BWE | OA | 0.2 | CA | 1:64 | 0.2 | 12.6 | 2.5 | 10.1 | 50 |
| | | | | | | | | mean | 68 |
| Anti-ovalbumin serum | | | | | | | | | |
| R4B | - | 0.1 | OA | 1:64 | 0.1 | 5.6 | 1.8 | 3.8 | 38 |
| R4B | - | 0.4 | OA | 1:64 | 0.4 | 25.2 | 7.2 | 18.0 | 45 |
| | | | | | | | | mean | 41 |
| Anti-twice recrystallized ovalbumin serum | | | | | | | | | |
| BWC | - | 0.2 | OA | 1:256 | 0.2 | 4.2 | 0.94 | 3.3 | 16 |
| BWC | - | 0.2 | CA | 1:64 | 0.2 | 5.6 | 2.5 | 3.1 | 15 |

filter (A. Gallenkamp and Co. Ltd.) in a centrifuge.

Other materials and equipment were sterilized by boiling, autoclaving, or with dry heat as appropriate. Chemical sterilizers and preservatives were not used.

10. Preparation of water-in-oil emulsions

A number of different methods and materials were used to form water-in-oil emulsions for the earlier experiments. Some of these were inefficient or difficult to carry out and they are described with the experiments in which each was used.

In all other experiments the following technique was adopted:

The mineral oil Drakeol 6-VR and the emulsifier Arlacel A were mixed together in the ratio of nine parts by volume of oil to one part of emulsifier and then sterilized by Seitz filtration.

The antigen was dissolved in saline and likewise Seitz filtered.

One volume of the oil/Arlacel mixture was placed in a suitable vessel such as a 25 ml 'Universal' screw cap container. An equal

volume of the antigen solution was available in another container.

A small portion of the antigen solution, say one fifth of its volume, was taken up in a disposable hypodermic syringe and violently squirted into the oil through a 0.5 mm needle, the point of which was held below the surface of the oil. The container was then capped and shaken vigorously before another portion of antigen solution was added in the same way. After the second or third addition of antigen, the mixed emulsion was taken up into the syringe through a wide bore (1 mm) needle and squirted out again through the fine bore (0.5 mm) needle used initially. More portions of the antigen solution were added and the process repeated until all of the antigen had been incorporated into the oil.

The dispersion of the water phase within the oil could be increased by repeatedly squirting the emulsion through 0.5 mm or 0.6 mm needles. This became very hard work as the viscosity of the emulsion increased with the increasing dispersion of the water phase. Some makes of syringe broke up under the pressure that it was necessary to exert.

Dispersion of the water phase could also be

increased by ultrasonication with a M.S.E. probe, but the emulsions produced were so viscous that they did not flow at all. They were, however, useful for further treatment to form multiple emulsions.

A more useful method was to make the emulsion with a syringe as described above and then pass it through a modified 'Bel' cream maker (Royston (Bel) Sales Ltd., Riversite Buildings, Erith, Kent). This is a kitchen implement used for making 'cream' from melted butter and milk. To adapt it for smaller quantities, three of the four intake holes were blanked off and a funnel attached to the fourth. This funnel embraced the top of the barrel so that emulsion which leaked past the piston was not lost but returned to the emulsifying valve (figure 3). The metal parts of the machine (after modification the emulsion only came into contact with these) could be sterilized by boiling. When assembled, the machine was held in a laboratory clamp and the output from below passed directly into a sterile container.

For most of the experiments syringes alone were used to prepare the emulsions. If ultrasonication or the 'Bel' cream maker were used, this is specially noted.

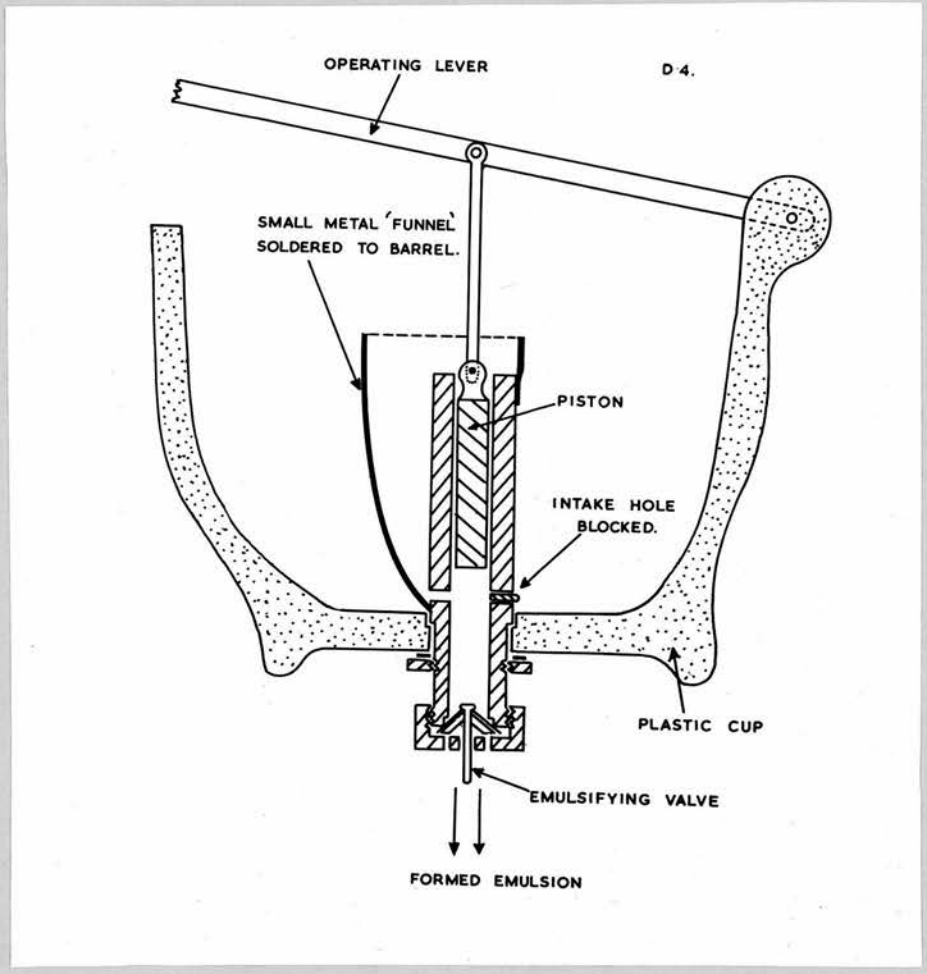



Fig.3. Diagrammatic cross-section through the modified 'Bel' cream maker used to prepare water-in-oil and multiple emulsions.

Before use, all water-in-oil emulsions were tested by allowing a portion to drop into iced water. If they were true water-in-oil emulsions they remained as discrete drops in this and did not disperse.

Emulsions were used within a day of preparation, unless otherwise stated.

## 11. The adsorption of ovalbumin on to aluminium hydroxide

An aluminium hydroxide precipitated ovalbumin preparation was made so that this type of depot forming adjuvant could be compared with the water-in-oil emulsion adjuvant.

Optimum conditions for the adsorption of protein on to aluminium hydroxide gel were determined by the method of Dr. Albert Hansen of the Danish State Serum Institute, Copenhagen, as described in a letter from Dansk Svovlsyre-OG, Superphosphat-Fabrik, the manufacturers of 'Alhydrogel' (1964). The method was altered slightly so that small quantities of material could be used.

Ovalbumin was dissolved in distilled water at a concentration of 1.25 mgm per ml. One ml of this solution was put into each of 12 plastic Wassermann tubes. Distilled water in amounts

decreasing in 0.1 ml steps from 1.1 to 0 ml was then added to each tube in the row.

The aluminium hydroxide gel was diluted 1:10 with distilled water and added to the tubes in volumes increasing in 0.1 ml steps from 0.1 to 1.2 ml. Each tube now contained a total of 2.2 ml.

After inversion to mix the contents, the tubes were allowed to stand undisturbed on the bench. Flocculation rapidly occurred in two of the tubes leaving a completely clear supernate within 30 minutes. Slight flocculation was observed in the tubes on either side of them.

To obtain this good end-point it had been necessary to repeat the titration twice, decreasing the concentration of ovalbumin each time. In the earlier attempts flocculation occurred first in the tube containing most aluminium hydroxide. It took rather longer to appear and most of the other tubes flocculated at about the same time.

Using the proportions of protein and aluminium hydroxide shown to be optimum, a batch of material for inoculation was prepared.

To 25 mgm of ovalbumin dissolved in 26 ml of distilled water, 18 ml of aluminium hydroxide gel (stock solution diluted 1:10 with distilled

water) was added. The mixture was allowed to flocculate overnight. It was then centrifuged at 500 G for 30 minutes and the supernate removed.

The pellet was made up to 2.5 ml with saline and well resuspended. A dose of 0.2 ml of this suspension was equivalent to 2 mgm of ovalbumin.

12. Inoculations, Collection of blood samples, Separation and preservation of sera

a) Inoculations

(i) Rabbits

Subcutaneous inoculations in rabbits were made in the loose subcutaneous tissue over the back.

(ii) Mice

Subcutaneous inoculations

All subcutaneous inoculations in mice were made in the dorsal/lateral area of the back in the loose subcutaneous tissue.

Inoculations of oil emulsions were made with disposable 2 ml plastic syringes through a 0.5 mm x 16 mm needle. Other solutions in saline solution were injected with a 'Plim' type 1 ml tuberculin syringe with an adjustable stop (Arnold and Sons, 54 Wigmore Street, London W.1) using No. 17 x ½ inch intradermal short point Schimmel needles.

Intravenous injections

Intravenous injections were made into the tail veins of mice with an all-glass 1 ml tuberculin syringe and a number 20 x $\frac{5}{8}$ inch needle. The mice were held in a small perforated zinc cage with the tail protruding. The veins were dilated by the application of warm water on a pledget of cotton wool before the injection was made.

Mice were not anaesthetized for either the subcutaneous or intravenous injections.

The injection of very small volumes of water-in-oil emulsion into lymph nodes

Very fine pipettes were made by drawing out 1.5 mm diameter capillary tubing in a microburner flame and separating the two pipettes thus formed by melting the connection.

For use, a pipette was thrust through a hole made with a needle in the top of an ordinary red rubber pipette teat. After being opened by breaking off the seal to leave a square cut tip, a very small amount of emulsion was drawn up into the narrow part of the pipette by manipulating the bulb whilst the normal opening was closed by one finger.

After initial anaesthesia in a jar, the mouse to be operated on was placed on the bench

and anaesthesia continued by allowing its muzzle to rest in a vial containing an ether-soaked pad.

With the animal lying on its right side the inguinal lymph node (Dunn, 1953) was located by palpation. The hair over a small area of the flank skin fold anterior to it was clipped away with wetted scissors. A fold of skin was then picked up and an incision made with the scissors. It was found to be best to make this incision run in an anterior-posterior direction and not across the body.

By manipulation with the fingers of the left hand, the lymph node was brought up to the incision and partly extruded through it. The tip of the pipette was then pushed into the node and a portion of its contents injected. It was sometimes possible to see the emulsion form a deposit within the node.

The lymph node was then allowed to slip back in place and a small quantity of a sulphonamide-urea-gentian violet powder (Cooper's Wound Dressing Powder) dusted on to the incision.

By weighing full and empty pipettes it was estimated that between 0.5 and 1 mgm of water-in-oil emulsion was injected into each node.

b) Collection of blood samples, separation and preservation of sera

(i) Human

Human blood samples were collected with a 10 ml syringe and allowed to clot overnight at room temperature. The sera were then removed and stored at -30°.

(ii) Rabbit

Rabbits were bled from the marginal ear vein. They were made to sit in a box of internal dimensions 5 x 5 x 16 inches with a moveable partition. In a quiet room no other restraint was found to be necessary. The hair over the vein was shaved, a thin smear of petroleum jelly applied, and a diagonal incision made across the vein. The base of the ear was then compressed with the left hand. An ample flow of blood was usually obtained if the rabbit was allowed to settle down quietly for a few minutes. Only rarely was it necessary to apply any benzole to the ear to encourage vasodilation. Up to 50 ml of blood was collected at one time.

Pressure over the cut with a small cotton-wool swab stopped the blood flow very readily if benzole had not been used.

The blood was collected into very clean 25 ml Universal containers and clotting had

usually occurred by the time of return to the laboratory. The containers were then placed in the 37° water bath for 30-60 minutes to cause rapid clot contraction. A check was made after 15 minutes or so to ensure that separation of the clot from the container walls was taking place. If this was not adequate, the clot was ringed by shaking.

When contraction was judged to be complete the clot was removed by hooking it out with a swab stick. The remaining red corpuscles were spun down by centrifugation at 1000 G for 10 minutes and the supernatant serum removed. It was stored ~~and~~ frozen at -20°.

If the interior surface of the Universal container was coated with a thin layer of agar, clot retraction was cleaner and fewer suspended cells were left. A 1 per cent solution of agar in saline was made by boiling. A portion of this was poured into a cold Universal container and out again quickly so that only a very thin layer remained. The containers were then capped and stored on the shelf until required.

(iii) Mouse

Mice were bled from the retro-orbital venous plexus by the method of Halpern and Pacaud (1951). They were not anaesthetized for

this procedure.

Miniature glass pipettes were used (as described elsewhere) and about 0.2 - 0.3 ml of blood was collected from each mouse. After collection the blood was gently expelled from the pipette into a Durham tube and this was stoppered with a natural cork on which the identity of the specimen had been noted. The pipettes were immediately dropped into a plastic beaker of cold water to lyse the blood remaining adherent to them. No anti-coagulants were used as serum samples were wanted. Only very rarely was any trouble experienced with clotting in the pipette during collection. If this did happen a fresh pipette was used to make another collection from the other eye.

Individual mice were bled up to 24 times over a period of 18 months without hurt. In the course of the present work about nine thousand blood samples were collected by this method without death or apparent injury to a single mouse.

The blood samples were allowed to clot and retract overnight at room temperature. In the morning the clots were removed by hooking them out with a bacteriological wire which had a small right angle bend at the tip. This wire was

flamed between each specimen. In some cases, when the clot was still adherent to the walls of the tube, it was found best to break up the clot rather than attempt to remove any of it and lose much of the serum as well.

After clot removal or break-up, the Durham tubes were packed into small plastic cups and centrifuged at about 1000 G to bring down the remaining red cells. These plastic cups were made from the bottom of a polythene bottle which would just fit into a large metal centrifuge bucket. A string was attached to the cup to enable it to be easily drawn out of the bucket.

After centrifugation, the serum supernate in each Durham tube was removed with a fresh miniature pipette and put into a clean tube. The cork bearing the identification number of the blood sample was transferred to the new tube. A clean pipette was used to transfer each sample.

The serum samples were stored at $-20^{o}$ in old cardboard slide boxes subdivided into small compartments. Identification of the individual samples was easy as the tubes were stored upright so that the numbers and a colour coding on the corks could be seen.

13. The preparation of tanned and coated sheep red cells

Most titrations of serum antibodies were carried out by the passive haemagglutination test of Boyden (1951). Fresh or formalinized sheep red cells were used. They were washed and then treated with a very dilute solution of tannic acid. After washing again, the cells were mixed with a solution of antigen some of which became adsorbed on to the cells. They thus became agglutinable by antibody against the antigen.

Due to the pre-treatment with tannic acid, these cells would give an agglutination pattern when allowed to settle out in a test tube even if no antibody was present. The addition of normal rabbit serum as a stabilizer prevented this non-specific agglutination.

Washing after coating was therefore carried out with dilute normal rabbit serum and the cells were finally suspended in this for use. When formalinized cells were used, the normal rabbit serum could be omitted until the last stage without harm. A test was then made to choose the best concentration for stabilization. It was found that fresh cells became irreversibly agglutinated unless normal rabbit serum was used

in all the washes done after coating.

Details of the methods used to prepare small and large volumes of cells and to formalinize the cells are given below.

a) Materials

(i) Fresh sheep cells

Fresh sheep cells from defibrinated blood were washed three times in 0.85 per cent sodium chloride (saline). They were packed at 750 G for 15 minutes before the volume required was measured out with a pipette.

(ii) Formalinized sheep red cells

Fresh sheep blood was collected from the abattoir and defibrinated by shaking in a bottle containing a wire spiral. The cells were then washed five times with phosphate buffered saline (P.B.S.) before being formalinized by the method of Csizmas (1960). During the formalinization process it was found convenient to have the cell suspension in a flask placed in a wire basket attached to a 'Matburn' mixer. The rotary movement imparted by this machine kept the contents of the flask well mixed without producing too much foam.

After formalinization the cells were washed seven times with saline. Sometimes several of

the washes were carried out with tap or distilled water without any deleterious effects. After washing, the cells were made up as an approximately 25 per cent suspension in saline. The exact strength of the suspension was checked by spinning down a portion in a haematocrit tube at 2000 G for 30 minutes. The suspension was stored in the refrigerator at $4^{o}$. For use, the volume of suspension calculated to give the wanted packed cell volume was measured out. The cells were spun down from this and washed once with saline.

(iii) Phosphate buffered saline (P.B.S.)

The following salts were dissolved in 1 litre of distilled water:

| | | |
|---|---|---|
| NaCl | 36 | g |
| $Na_2HPO_4$ | 7.4 | g |
| $KH_2PO_4$ | 2.15 | g |

The solution was stored in the refrigerator at $4^{o}$. For use, it was diluted 1:5 with distilled water. The pH was between 7.2 and 7.4.

(iv) Tannic acid

The tannic acid solution was freshly prepared for each batch of cells. It was a 1:10,000 solution of tannic acid (Mallinkrodt Chem. Corp.) in P.B.S. (2.5 mgm in 25 ml).

(v) Antigen

Protein antigen for coating the cells was prepared as a 0.2 per cent solution in P.B.S. (20 mgm in 10 ml).

(vi) Normal rabbit serum (N.R.S.)

Normal rabbit serum was inactivated at 56° for 30 minutes and then absorbed with cells of the batch to be coated. Packed cells equivalent to one fifth or one tenth of the volume of serum to be treated were added to it and absorption carried out at room temperature for at least 30 minutes. It was found advantageous to do this with gentle agitation on a 'Matburn' mixer. The cells were removed by centrifugation.

A single absorption was usually sufficient, but when time was available, the cells were absorbed a second time. This made quite sure that no agglutinins remained to spoil the settling patterns of the cells.

b) Tanning and coating small (50 ml) quantities of fresh or formalinized cells

All manipulations were carried out in 1 oz screw cap vials (McCartney bottles) which are convenient for use in a bench centrifuge.

(i) In each of two vials 0.6 ml of packed cells were suspended in 10 ml of P.B.S.

(ii) 10 ml of tannic acid solution was rapidly

added to each and they were shaken up immediately. Both vials were then allowed to stand in the water bath at 37° for 15 minutes.

(iii) The cells, now tanned, were spun down at 750 G for 5 minutes, the supernate discarded and the cells washed once with P.B.S. When re-suspending fresh cells for washing it was found best to add only 1-2 ml of P.B.S. at first and suspend them in this. If more P.B.S. was added the cell pellet tended to come off the bottom of the vial in a lump which was difficult to break up.

(iv) One vial was put aside to be used for the control cells which are tanned only. The cells in the other vial were resuspended in 10 ml of P.B.S. and 10 ml of the antigen solution added. The whole was then quickly shaken up and placed in the 37° water-bath for 30 minutes. During this time it was gently shaken once or twice.

(v) After coating, the cells were spun down at 750 G for 5 minutes and the supernate discarded. The cells in both vials were then washed three times with 1 per cent normal rabbit serum in P.B.S. After the third wash they were resuspended to 50 ml each.

The suspension was stored in flat sided

screw cap medicine bottles at 4°, and in the case of fresh cells was used within three days of preparation. The final suspensions contained approximately 1 per cent of cells, it being assumed that about 0.1 ml of the 0.6 ml of packed cells taken to start with would have been lost during the various washes.

c) Tanning and coating large (500 ml) quantities of formalinized cells

The same methods and materials were used as are given above for the preparation of small volumes of cells.

The process was carried out in a 1 oz vial (McCartney bottle) but the packed cell volume of formalinized cells used was 6 ml. This quantity of cells made a deep and thick pellet after centrifugation so that resuspension had to be done by gentle stirring with a thin glass rod. Great care was taken to ensure that the cells were properly resuspended at each stage.

For tanning, the cells were suspended in sufficient P.B.S. to half fill the vial and 12 ml of P.B.S. containing 40 mg of tannic acid added. This addition was done very quickly and the whole vial was shaken vigorously immediately afterwards, stoppered only with a finger. Very rapid mixing of the cells and tannic acid was essential to the

successful preparation of tanned cells in bulk by this method. The cells were tanned at room temperature for 20 minutes, the vial being clipped to the 'Matburn' mixer so that they were continually agitated during this period.

After tanning, the cells were washed once with P.B.S. and resuspended to half fill the vial. Twenty milligrams of ovalbumin in 12 ml of P.B.S. were added and the whole mixed quickly. The vial was then placed for 45 minutes on the 'Matburn' mixer at room temperature.

After coating, the cells were washed three times with P.B.S. and then made up to 500 ml in P.B.S. Portions of the suspension were tested in a doubling dilution series of normal rabbit serum in saline and the highest dilution giving a good negative button was noted. Sufficient normal rabbit serum to bring the whole suspension to this dilution was then added to it.

Whilst the above test was being carried out, 10 ml of suspension stabilized with normal rabbit serum at a dilution of 1:100 was tested with an anti-ovalbumin serum. This was to ensure that the cells were working properly before they were frozen.

d) The preservation of tanned and coated formalinized cells, by freezing

The completed suspension was distributed, in 10-20 ml quantities, to 1 oz vials (McCartney bottles) or Universal containers and the caps screwed on tight. Some material was also put in Wassermann tubes so that small quantities of frozen cells were available for test and standardization purposes. These were stoppered with rubber corks.

The bottles were shaken to ensure complete suspension of the cells and quick freezing was carried out by immersing the bottles in a mixture of dry ice and acetone until the contents were solid. The bottles were then placed in the deep-freeze cabinet at $-20^{o}$ for long term storage.

e) Standardization of each batch of fresh or formalinized cells

Each batch of cells tended to vary in its sensitivity to antibody. Each batch was therefore compared with standard material so that if necessary a correction could be made to all serum antibody titres measured with it.

Doubling dilutions of a mouse anti-ovalbumin serum were made in 1 ml volumes in saline. Portions of each dilution in this series were

then distributed in 0.1 ml volumes to the wells in a plastic agglutination plate. Thus the 1:1280 dilution in each row of wells would be comparable even though they might not really be dilutions of exactly 1:1280. To the wells in each row were added 0.1 ml portions of batches of cells of known and unknown sensitivity. The plate was shaken well and left on the bench overnight before the end-points were read. It was then possible to compare accurately the relative sensitivities of the cells and these were noted for reference.

14. The titration of sera by the tanned cell technique

a) General method

Firstly it was necessary to absorb out any agglutinins against the sheep cells themselves which would have given erroneous non-specific titres. Sera were diluted 1:10 with a 1 per cent suspension of tanned but uncoated cells. They were then allowed to stand on the bench for 1-2 hours, with occasional shaking, before the cells were removed by centrifugation. The sera were not inactivated before titration.

Doubling dilutions of the supernate were made in 0.1 ml volumes in a W.H.O. plastic plate

using an automatic 1 ml syringe (R.B. Turner and Co., London N.2) and miniature Pasteur pipettes. An extra well was prepared with 0.1 ml of the 1:10 dilution. In the test, uncoated cells were added to this well as a control to check that all non-specific agglutinins had been removed.

Coated cells in 0.1 ml volumes were added to each of the wells containing the doubling dilution. The plate was shaken well and left on the bench for 2 hours in the case of fresh cells, or overnight when formalinized cells had been used, before the titres were read. If evaporation was prevented, there was very little change in end-point position if these times were extended. If necessary, the cells could be re-suspended and allowed to form fresh settling patterns without any change occurring in the position of the end-point.

The settling patterns were read by taking a complete coating of the bottom of the well as +++, a neat round button as - and the intermediate points as ++, + and $\pm$. The end-point was then the + point. In the experiments reported in the present work, numerous titrations were carried out at the same time. It was therefore quite easy to choose a suitable pattern to be taken as the end-point by examining a whole

plate of titrations before starting to record the results.

Sera were always set out in a random order on the plates to minimize any subjective influence on end-point reading due to knowledge of the experiment being conducted.

b) Modifications of the general method; used when mouse sera were being titrated

(i) To ensure the complete removal of non-specific agglutinins, it was found preferable to absorb all sera with a 2 per cent suspension of untanned cells when formalinized cells were to be used for the test.

When very low titres were expected, fresh cells were used to titrate the sera as these cells were usually more sensitive. It was then found convenient to make sure that any non-specific agglutinins were eliminated by making the initial 1:10 dilution with one drop of untreated packed cells, eight drops of 1 per cent tanned but uncoated cells and one drop of serum.

(ii) Initial dilution and absorption was carried out in Durham tubes (22 mm x 6 mm). Dropping dilutions were made using the miniature pipettes described elsewhere. The cell suspension was measured out first, the tip of the pipette wiped and then one drop of serum added

from it. The pipette was then discarded to be washed. The tubes were corked (the reference number being marked on the cork) and left on the bench for 1-2 hours for absorption to take place. They were inverted to mix the contents several times during this period. The cells were brought down by centrifugation before the supernate was titrated.

(iii) With some very low titred sera, initial dilutions of 1:2, or 1:3, etc., were made by the dropping method. The actual dilution prepared depended on the amount of serum available and the necessity of having at least 0.3 ml of diluted material available to supply the initial dilution, start of the doubling dilution, and the control well.

15. Haemagglutination inhibition tests for antibodies against influenza virus

a) Human sera

Haemagglutination inhibition tests for antibodies in human sera were carried out by the standard plastic plate method (W.H.O., 1953) using 4 minimum haemagglutinating doses (M.H.D.) of the appropriate strain of virus. The last well showing complete agglutination was taken as the end-point in the virus titrations. The last

well showing complete inhibition was taken as the end-point in the serum titrations. Fowl cells were used as the indicator.

Before titration, non-specific inhibitors of viral haemagglutination were removed by overnight treatment with cholera filtrate (R.D.E.) obtained from Burroughs Wellcome and Co. The residual R.D.E. was inactivated by heating to 56° for one hour before titration.

b) Mouse sera

The standard method was followed but the mouse sera were titrated in 0.1 ml, instead of 0.25 ml volumes. The diluted virus and the fowl cells suspension were also added in 0.1 ml volumes. The fowl cells were used as a 1 per cent suspension instead of the 0.5 per cent suspension used for the human sera. If the more dilute suspension was used it was found that there were insufficient cells to give adequate settling patterns. The virus was titrated in these same small volumes and with the 1 per cent cells so that 4 M.H.D. were added to each well in the test proper, in the usual way.

Heating to 56° or treatment with cholera filtrate did not remove the non-specific inhibitors in mouse sera. The sera were there-

fore merely diluted with calcium saline (saline containing 0.1 per cent of calcium chloride; the diluant used in all of these tests) before titration.

16. Analysis of sera for IgM type antibodies

The immunoglubulins in serum are of at least two main types, IgG ($\gamma$, 7S$\gamma$, $\gamma_2$, $\gamma_{ss}$, etc.) and IgM ($\gamma_1$M, $\beta_2$M, 19S$\gamma$, etc.). The amount of each of these present varies during the course of the antibody response and they may thus be indicators of the type of antibody forming mechanism which is active at any one time.

As their immunological reactions are very similar, separation has to be carried out by physical methods. One way in which this can be done is to destroy the IgM type. Treatment of the serum with 2-mercaptoethanol achieves this by splitting the molecule at S-S bonds to leave inactive fragments. The unaffected IgG antibody titre can then be measured and hence the IgM titre by difference.

Another way of separating the two types of immunoglobulins is by gel filtration on the cross-linked dextran preparation Sephadex G200. This is packed into a column and a buffer containing

the serum to be separated allowed to flow it. The smaller IgG molecules enter the gel structure and are retarded. The larger IgM molecules cannot enter, flow faster through the column and appear first in the eluate.

Both methods of separation were used in the present work.

a) Treatment of sera with 2-mercaptoethanol to destroy IgM antibodies

The method of Deutsch and Morton (1957) was followed; they treated a 1 per cent solution of protein with 0.1 M 2-mercaptoethanol.

The serum to be treated was therefore diluted 1:3 with phosphate buffered saline (P.B.S. prepared as in Method 13) and an equal quantity of 0.2 M 2-mercaptoethanol added. The 0.2 M solution was made by diluting 1.9 ml of 2-mercaptoethanol (Light and Co. Ltd.) to 100 ml with P.B.S.

A control for each serum treated was set up. In these the mercaptoethanol was replaced by saline.

All manipulations were carried out in a fume cupboard. The tubes containing the treated serum and the control tubes were then stoppered and they were left at room temperature overnight. In the morning each serum was put into dialysis

tubing and placed in a beaker of P.B.S. to dialyse away the excess mercaptoethanol. The controls were put into a separate beaker to ensure that they were not affected by mercaptoethanol, but otherwise they were treated in exactly the same way throughout.

Dialysis was carried out over a period of 10 days at $4^{o}$ during which time the P.B.S. was changed five times. The sera were then removed from the dialysis tubes, put into Wassermann tubes and frozen at $-20^{o}$ for storage prior to titration. The volume of each serum recovered was very much the same and no adjustments were made.

Before titration with fresh tanned sheep cells, samples of the treated sera and of the controls were measured into Durham tubes and one drop of packed sheep cells added to each. The tubes were then stoppered, shaken up several times and left at $4^{o}$ overnight in order that any agglutinins for sheep cells present might be absorbed out.

b) Separation of IgM and IgG type antibodies on a Sephadex G200 column

The method used followed in general that of Flodin and Killander (1962).

A glass tube about 2 metres long by 4 cm

wide was prepared to hold the Sephadex. It was stoppered at one end with a rubber cork through which ran a narrow glass outlet tube. Inset into the upper side of the cork was a perforated zinc disc supporting six layers of surgical gauze to retain the Sephadex. A single layer of glass beads prevented disturbance of the gauze during filling procedures.

About 20 grams of Sephadex G200 (Lot TO 3017, Pharmacia, Upsala, Sweden) was allowed to swell in 0.2 M tris-HCl buffer (pH 8.0) diluted in 0.2 M NaCl (Cruickshank, 1960) for three days. It was then well de-aerated using a filter pump, and poured into the glass tube which had already been partly filled with buffer. The whole contents of the column were well stirred up and allowed to settle for a short while before any buffer was allowed to run from the outlet at the bottom.

Buffer was then allowed to flow very slowly overnight whilst the Sephadex settled and compacted. The Sephadex level was marked on the outside of the tube at intervals until it stabilized. The height of the column was then 67 cm, corresponding to a volume of 3370 ml.

The serum to be treated was layered on to the Sephadex surface with the aid of a 1 ml

tuberculin syringe attached to a length of narrow polythene tubing (3 mm external, and 2 mm internal diameter). The outlet end of this tubing had been constricted a little in a Bunsen flame.

Syringe and tubing were completely filled with buffer, care being taken to eliminate any bubbles. With the syringe clamped to a convenient support and the serum container held outside the tube at the level of the Sephadex surface, about 1 ml of serum was drawn into the tubing. The tube was then passed into the top of the column and down through the overlying buffer until it was three or four centimetres above the Sephadex surface. The plunger of the syringe was pushed in very gently whilst the tip of the polythene tube was moved about a little, so that a layer of serum was deposited over the Sephadex surface without disturbing it. Buffer was not allowed to run through the column whilst this was being done.

When the layering had been completed the column outlet was opened and more tris buffer added to fill up the long column as soon as the serum layer was seen to enter the packing.

The flow rate from this column with the outlet unrestricted, and the upper part of the

tube filled with buffer, was about 40 ml an hour. Fractions were collected in an automatic fraction collector (Locarte Company, London S.W.7) using a drop counter.

The protein content of the fractions was estimated in an ultra-violet spectrophotometer and recorded as optical density at 280 mμ. A curve was drawn from these measurements and the fractions constituting each peak were pooled. These pools were put into Visking dialysis tubing and reduced almost to dryness by surrounding them with polyethylene glycol (mol. wt. 6000, L. Light and Co. Ltd., Colnbrook) at 4°.

Each pool was then reconstituted to the original serum volume by the addition of distilled water and they were stored at -20° until titration was carried out.

17. Statistical methods used

Student's t tests were carried out by the methods described by Croxton (1959) and Simpson, Roe, and Lewontin (1960). This test measures the extent to which it is probable that two groups of results come from the same population of possible results. It should be noted that the method involves the comparison of the

arithmetic means of the results for each group. In the tables of results and graphs presented, geometric mean titres are used.

The geometric mean gives a lower figure than the arithmetic mean but is less affected by extreme values or the occasional apparently aberrant reading which occurs in work of this type. There is therefore no need to make an arbitrary discard of such readings. Another advantage is that the 'centre of gravity' of the geometric mean is relatively difficult to move. Curves drawn from geometric means are therefore smoother than those drawn from arithmetic means of the same results. In all the graphs presented below, the plotted points have been directly joined, smoothed curves were not fitted for them.

# Part Three

## Experimental

## PURIFICATION OF THE ANTIGEN

# EXPERIMENTAL

## PURIFICATION OF THE ANTIGEN



### A. INTRODUCTION

Crystalline ovalbumin was for long considered to be one of the purest proteins

available. Physical and chemical studies substantiated this proposition but immunological methods of analysis have shown that it is not free from a number of other egg-white proteins (Munoz and Becker, 1950).

Absolute purity of any substance, organic or inorganic, is difficult to achieve, and in many cases the presence of a minute quantity of some other material is not important, unless this impurity has some extremely potent biological function. None of the proteins found in egg-white, except perhaps lysozyme, can be considered to be in this category and for most purposes crystalline ovalbumin can be treated as a single material.

However, Borduas and Grabar (1953) reported anomalous results when crystalline ovalbumin was used as the coating antigen in the very sensitive tanned red cell agglutination test of Boyden (1951). These were possibly due to the presence of another egg-white protein conalbumin. Borduas and Grabar believed that, not only was the conalbumin a more potent antigen than ovalbumin but that it was preferentially adsorbed on to the red cells. Thus the titre of an anti-ovalbumin serum measured by this method might in fact be the titre of a trace of anti-conalbumin

in it.

Ovalbumin had been chosen as the chief antigen to be used in the adjuvant experiments. The antibodies raised against it could be measured most conveniently by the tanned cell technique. It was therefore necessary to re-investigate Borduas and Grabar's findings; confirmation would mean, either that conalbumin-free ovalbumin must be used in all the investigations, or that another antigen must be sought. In any case at least a small quantity of very pure ovalbumin would have to be prepared for the investigation.

Following the work of Sørensen in 1934 it is generally agreed that in egg-white there are five major proteins from which the ovalbumin would have to be separated. These are:

a) The simple proteins

| | |
|---|---|
| Ovalbumin | 75% of total protein |
| Conalbumin | 3% |
| Ovoglobulin | 2% |

b) The glycoproteins

| | |
|---|---|
| Ovomucoid | 13% |
| Ovomucin | 7% |

The usual method of extracting ovalbumin from egg-white is by salting out and crystallization using either ammonium sulphate (Sørensen,

1915), or sodium sulphate (Kekwick and Cannan, 1936). Commercially available ovalbumin has usually been recrystallized two or three times. Consideration was given to the possibility of preparing a much purer sample by repeated recrystallizations but Stavitsky (1954b) has shown that even after seven recrystallizations ovalbumin is still contaminated with conalbumin.

Other methods of preparation are: electrophoresis in a starch block (Kunkel, 1954), and ion exchange chromatography (Rhodes, Azari, and Feeney, 1958; Mandeles, 1960). The first would only be suitable for the production of minute quantities of material, but the second might be used on a much larger scale.

Rhodes et al. separated the egg-white proteins with carboxymethyl-cellulose (CM-cellulose). The egg-white was adsorbed on to this material at an acid pH and the proteins eluted off in sequence by a series of buffers of increasing alkalinity. Ovalbumin is not homogeneous, and Rhodes et al. were able to obtain separation of the ovalbumin itself into three components by this method. Mandeles used diethylaminoethyl cellulose (DEAE-cellulose) and buffers of varying molarities and composition. His results showed only two, poorly separated,

ovalbumin elution peaks.

The method of Rhodes *et al*. seemed very promising but a digression must be made here to elaborate further on ovalbumin itself. Early studies of the egg-white proteins by the moving boundary method of electrophoresis showed two peaks in the albumin region. These were called A1 and A2 (Tiselius and Ericksson-Quensel, 1939). Later investigations showed that there is a third peak A3 (MacPherson, Moore and Longsworth, 1944; Cann, 1949), and the most recent work using starch-gel electrophoresis suggests that there may even be a fourth in some cases (Lush, 1961).

MacPherson *et al*. (1944) found that the proportions of the different ovalbumins varied with time. Fresh albumen (egg-white) contained A1 in the greatest quantity but this changed entirely to A2 during seven months storage in salt-free solution. The change also occurred in ovalbumin dried to a powder, though more slowly. By enzymic degradation, Perlmann (1952) showed that the three ovalbumins differed in the number of phosphorus atoms per molecule. A1 having two atoms, A2 one atom, and A3 none.

In 1956 Durieux and Kaminski showed that the three ovalbumins were immunologically identical. After separation by electrophoresis in agar, they

were eluted and examined by double diffusion against an anti-ovalbumin serum. The precipitation lines formed with each ovalbumin linked up in a reaction of identity. The presence or absence of phosphorus atoms does not, therefore, appear to affect the antigenic determinants of the molecule.

Rhodes et al. reported that these three ovalbumins could easily be separated by the chromatographic method. Although no reference to the immunologic purity of ovalbumin prepared in this way could be found it commended itself as one which might produce, in quantity, a purer material than those that had hitherto been used.

## B. RESULTS

### 1. Examination of whole egg-white

As a preliminary to any other investigations, it was first necessary to make a presumptive identification of the components of whole egg-white revealed by electrophoresis, immunodiffusion, and immunoelectrophoresis. Where possible this was done by comparison with known materials.

#### a) Electrophoresis on cellulose-acetate paper

Diluted whole egg-white and a known sample of conalbumin (kindly supplied by Dr. I.E. Lush) were electrophoresed at the same time on one sheet of paper. Figure 4 shows that a strong conalbumin component in the egg-white could be identified and that the three ovalbumins were resolved.

Figure 5 shows the electrophoretic pattern of neat egg-white. The staining is irregular due to the high concentration of protein, but more components are shown. They are identified on the figure from the data of Lush (1961).

#### b) Immunodiffusion

In double diffusion tests, whole egg-white showed six lines when set up against hyperimmune

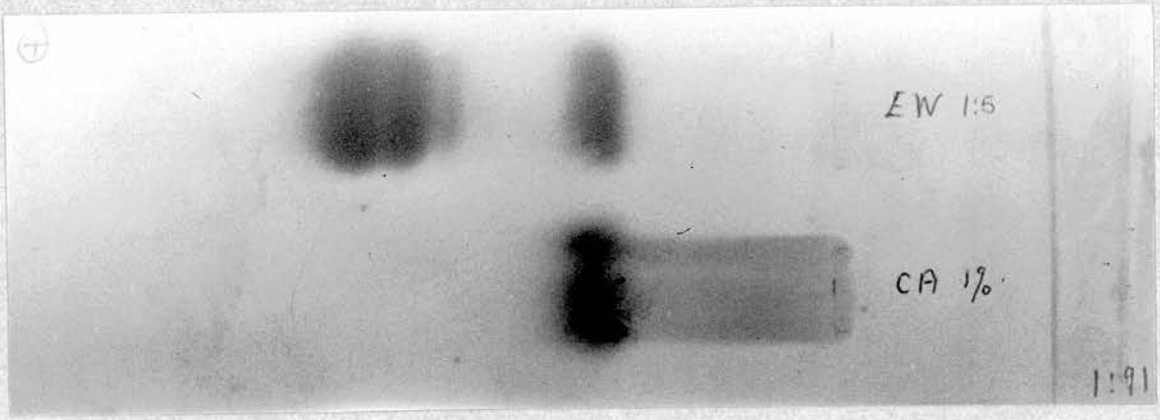



Fig.4. Electrophoresis on cellulose acetate paper.
Above: Whole egg-white diluted 1:5, showing the three ovalbumins (left) and a single conalbumin band (right).
Below: A known specimen of conalbumin (kindly supplied by Dr.I.E.Lush).
Electrophoresis carried out for 7 hours.

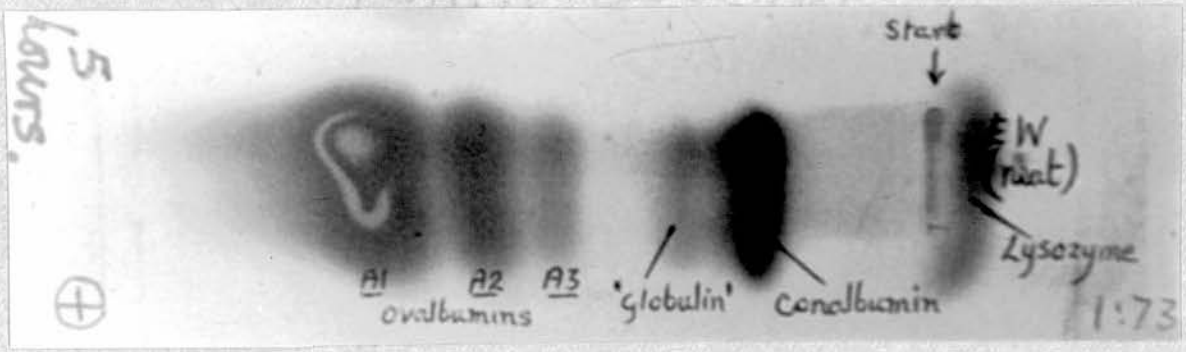



Fig.5. Electrophoresis of whole egg-white on cellulose acetate paper. The egg-white proteins revealed are labelled from the data of Lush (1961).

anti-egg-white sera. The heavy line near the antibody well in figure 6 can be identified with the ovalbumin, anti-ovalbumin system. It shows a reaction of identity with the line formed by a chromatographically prepared material with the electrophoretic characteristics of ovalbumin (fig. 7).

The other heavy line, nearer to the antigen well, shows a reaction of identity with the conalbumin system (fig. 8).

c) Immunoelectrophoresis

Figure 9 shows immunoelectrophoresis of whole egg-white, developed with anti-egg-white serum.

In the top slide the egg-white was accompanied by commercial crystalline ovalbumin. This enables identification of the ovalbumin line to be made. At the bottom, a known sample of conalbumin was run at the same time, and the line probably corresponding to the conalbumin system can be picked out.

2. Examination of the purity of crystalline ovalbumin

A commercial sample of twice recrystallized ovalbumin (L. Light and Co. Ltd., Colnbrook,

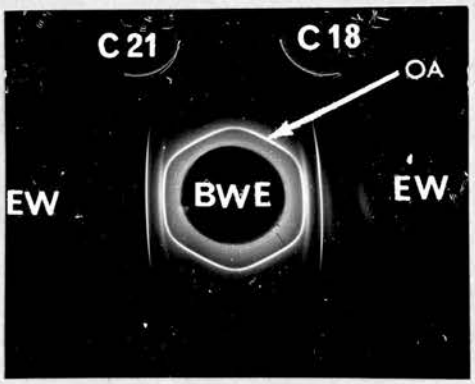



Fig.6. Immunodiffusion analysis of whole egg-white in P.B.S. agar.
B.W.E. = anti-egg-white serum.
E.W. = egg-white diluted 1:5.
C21 & C18 = chromatographically purified ovalbumin.
The arrow points to the precipitation line of the ovalbumin-anti-ovalbumin system.

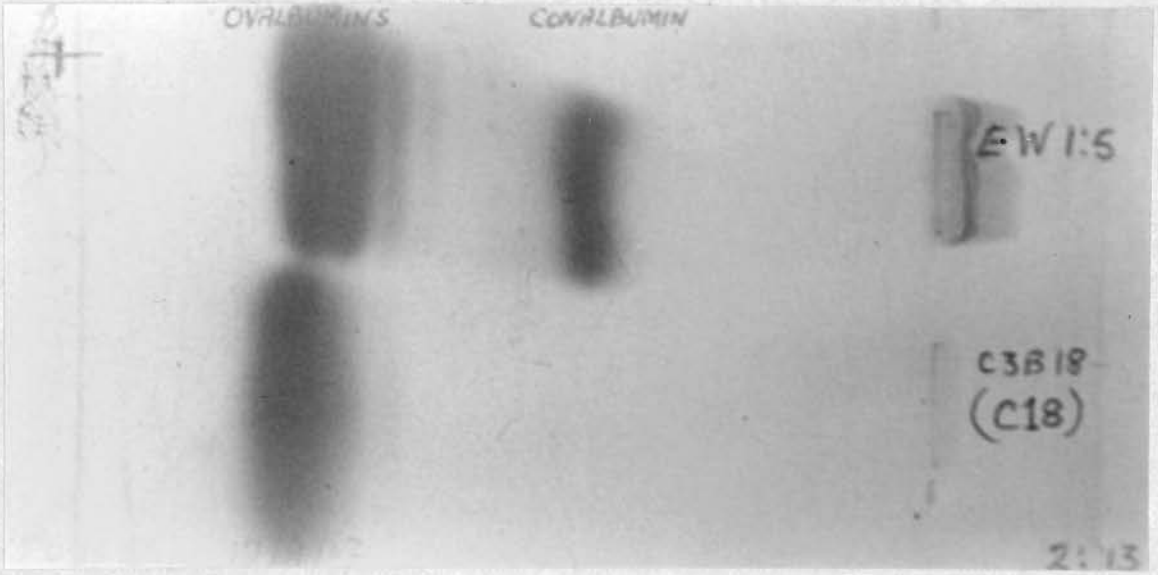



Fig.7. Electrophoresis on cellulose acetate paper.
Above: Whole egg-white diluted 1:5.
Below: Chromatographically purified ovalbumin batch C18.
Electrophoresed for 2 hours in a discontinuous buffer pH 8.65 as described by Poulik (1957).

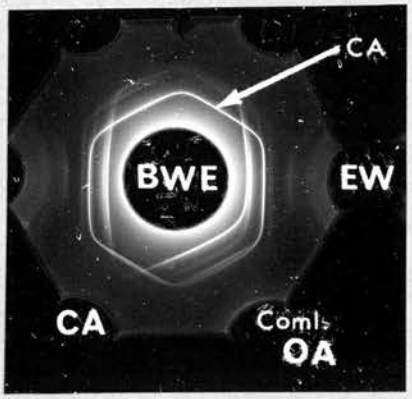



Fig.8. Immunodiffusion analysis of whole egg-white in P.B.S. agar.
B.W.E. = Anti-egg-white serum.
CA = Electrophoretically pure conalbumin (Dr.I.E.Lush).
EW = Egg-white diluted 1:5.
Coml OA = Twice recrystallized ovalbumin from a commercial source.
The arrow points to the precipitation line representing the conalbumin-anti-conalbumin system.

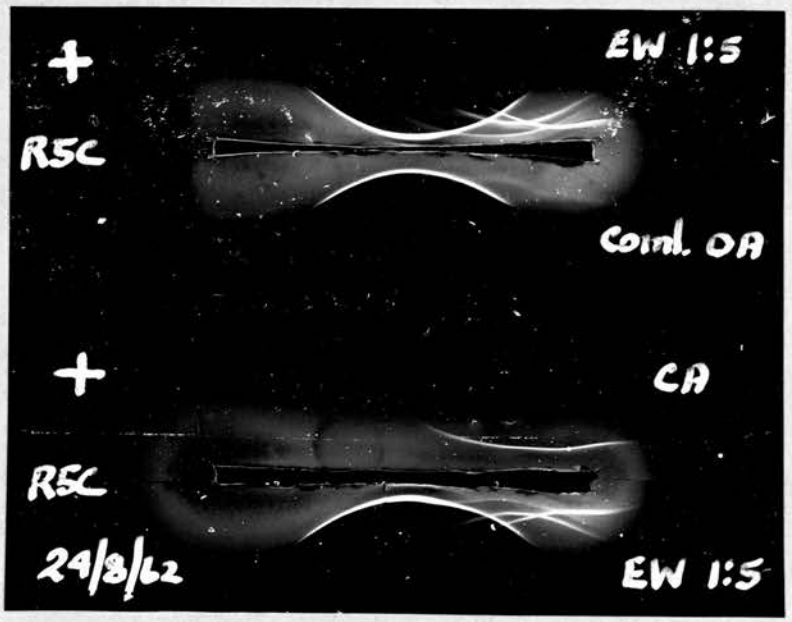



Fig.9. Immunoelectrophoresis of egg-white, crystalline ovalbumin, and conalbumin.
EW = Whole egg-white diluted 1:5.
Coml OA = Twice recrystallized ovalbumin from a commercial source.
CA = Electrophoretically pure conalbumin (Dr.I.E.Lush)
R5C = Anti-egg-white serum in the centre trough.

England) was examined to find out whether other antigens were present in addition to ovalbumin.

a) Electrophoresis on cellulose-acetate paper

The electrophoretic pattern of the crystalline ovalbumin is shown in figure 10. A slower moving component is present in addition to the ovalbumins. This component is in the same position as is pure conalbumin run on the same sheet.

b) Immunodiffusion

When set up in double diffusion against anti-egg-white sera (figs. 8 and 33), crystalline ovalbumin showed a very similar pattern to that given by whole egg-white. One of the lines in figure 8 shows a reaction of identity with that of a known conalbumin preparation.

c) Immunoelectrophoresis

Three components were resolved by immuno-electrophoresis developed by an anti-egg-white serum (fig. 9).

These tests showed that the recrystallized ovalbumin was very impure. It contained many of the antigens present in whole egg-white and in particular was rich in conalbumin. If,

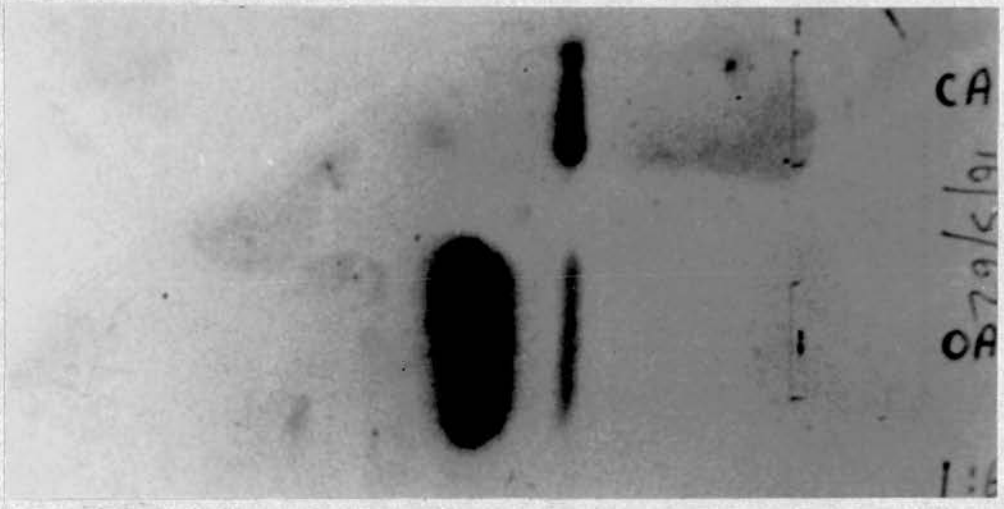



Fig.10. Electrophoresis on cellulose acetate paper.

Above: Electrophoretically pure conalbumin (Dr.I.E.Lush).
Below: Twice recrystallized ovalbumin from a commercial source. This shows a marked conalbumin band in addition to the ovalbumins.

Electrophoresis carried out for 2 hours.

therefore, conalbumin did interfere with the tanned cell test for anti-ovalbumin antibodies, ovalbumin prepared in this way could not be used to coat the cells.

### 3. The separation of egg-white proteins by elution from a carboxymethyl-cellulose column using stepwise pH changes

It has been noted in the introduction that Rhodes, Azari and Feeney (1958) separated the egg-white proteins by ion exchange chromatography on carboxymethyl-cellulose. Although they did not examine the immunological purity of the materials obtained, their method appeared to be one by which ovalbumin, free of conalbumin, might be prepared.

A column packed with carboxymethyl-cellulose was set up as shown in figure 1, and equilibrated to pH 4.2 with ammonium acetate - acetic acid buffer. Egg-white was prepared as detailed in the Methods section and 75 ml applied to the column.

Rhodes et al. (1958) found that peak elution of the egg-white proteins from CM-cellulose occurred at the following pHs:

| | |
|---|---|
| Ovomucoid and Flavoprotein | pH 4.3 |
| Ovomucoid | 4.4 |

| | |
|---|---|
| Ovalbumin A1 | pH 4.5 |
| Ovalbumin A2 | 4.65 |
| Ovalbumin A3 | 4.85 |
| 'Globulin' | 5.2 |
| Conalbumin 1 | 5.8 |
| Conalbumin 2 | 6.1 |

Other 'globulin' fractions and lysozyme were eluted at higher pHs.

This data was followed using slightly more alkaline buffers to ensure a good change-over between the steps.

The unwanted ovomucoid and flavoprotein were first removed by passing 200 ml of pH 4.2 and then 200 ml of pH 4.4 buffer through the column. As these were flowing a white band with a yellow flare below it was seen to pass down the column but another white band remained at the top. The eluates were discarded.

Elution of the wanted materials was carried out as described in the Methods section using the following sequence of buffers:

| Volume | pH |
|---|---|
| 250 ml | 4.56 |
| 250 ml | 4.70 |
| 200 ml | 4.90 |
| 150 ml | 5.30 |
| 200 ml | 5.60 |
| 200 ml | 5.90 |

and then sufficient 0.1 M ammonium acetate solution to run out the last buffer.

The eluate was collected in 20 ml fractions. The pH of each was measured, and its protein content determined by the biuret reaction.

The results were plotted and are shown graphically in figure 11. There was a very crisp separation of the different protein peaks. The A2 peak is higher than expected for ovalbumin A1 is claimed to be the major component in fresh material (MacPherson et al., 1944).

The fractions were pooled in groups as indicated by square brackets on the graph (fig. 11). Each pool was identified by the number of the first fraction in it. The pools were examined by electrophoresis and immunodiffusion.

a) Electrophoresis on cellulose acetate paper

The electrophoretic patterns of all the pools are shown in fig. 12a and b. That of one of the pools and egg-white is shown in fig. 13. These patterns show that only the first and last of the peaks are electrophoretically homogeneous. Despite the relative sharpness of the other peaks, they contain mixtures of proteins known to be antigenically different.

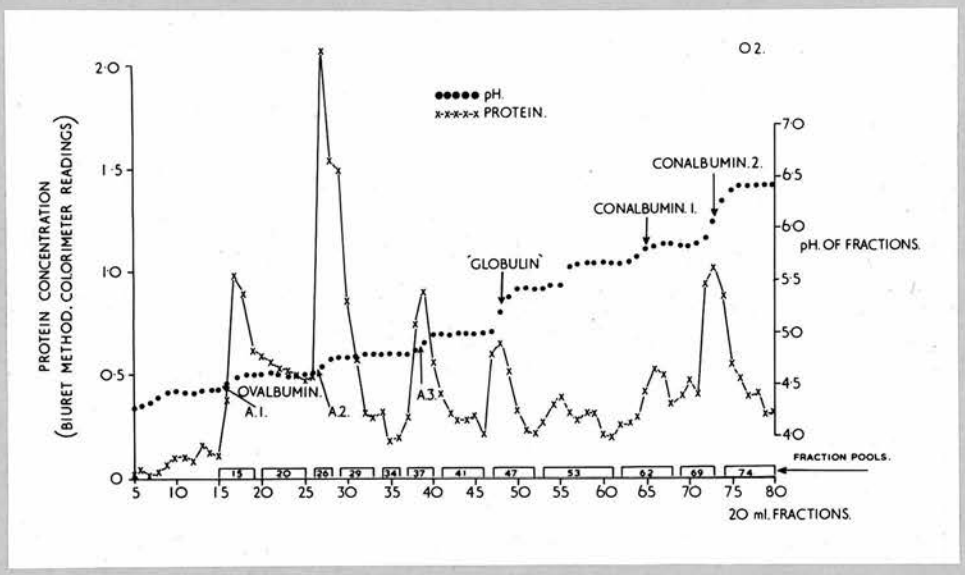



Fig.11. Separation of egg-white proteins by chromatography on CM-cellulose. Elution of the proteins by stepwise pH changes.

The protein content and pH of each fraction is indicated on the graph. The pH curve is marked at the points given by Rhodes et al. (1958) for the peak elution of each protein.

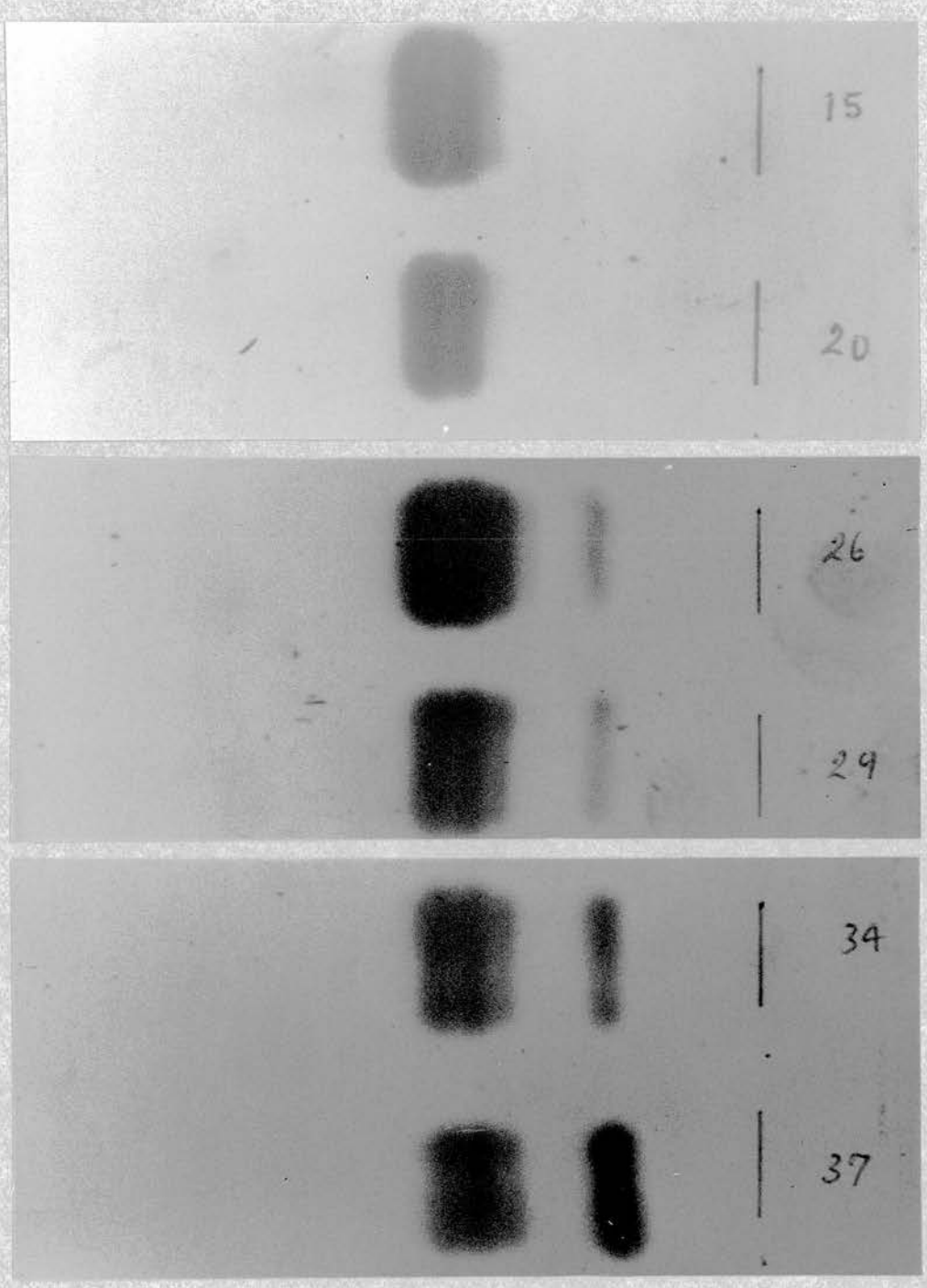



Fig.12a. Electrophoresis on cellulose acetate paper of egg-white proteins eluted from CM-cellulose by a stepwise increase of pH. The numbers refer to the fraction pools shown in fig.11. Pools 15 and 20 show ovalbumin only. The later pools contain increasing amounts of conalbumin.

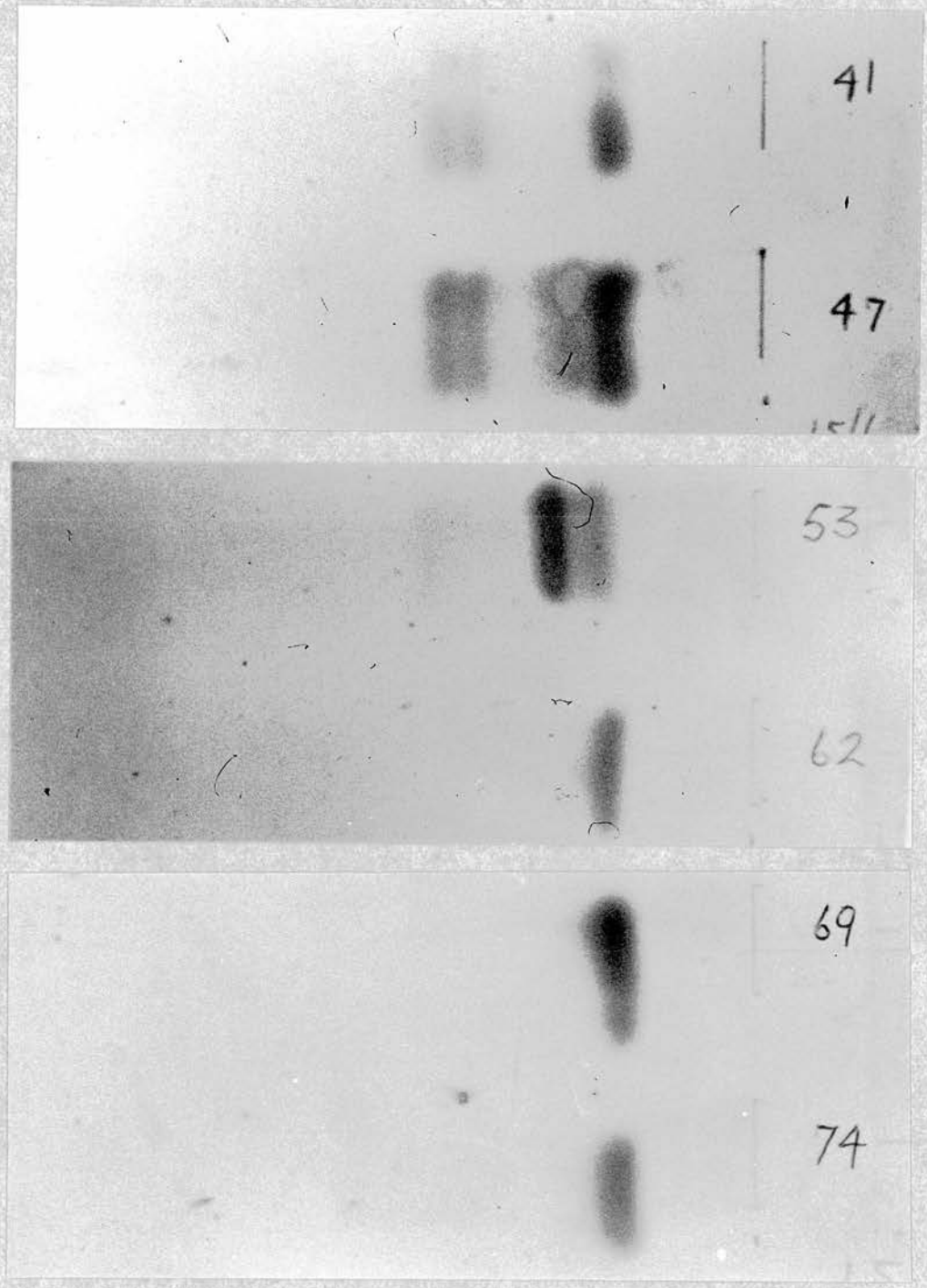



Fig.12b. Electrophoresis on cellulose acetate paper of egg-white proteins eluted from CM-cellulose by a stepwise increase of pH. The numbers refer to the fraction pools shown in fig.11. All the pools show conalbumin. Pools 41 and 47 show some ovalbumin. Pools 47 and 53 also show some 'globulin'.
Electrophoresis carried out for 2 hours.

b) Immunodiffusion

The pooled fractions were allowed to react with anti-egg-white serum by double diffusion through agar (fig. 14). This showed that all of the pools except No. 15 contained more than one antigen. The relative abundance of the proteins in each can be followed clockwise round the plates, those present in the greatest quantity giving lines nearest to the antibody well. The earlier pools contain ovalbumin. This decreases in quantity in each pool after No. 41 but conalbumin then appears in increasing amounts.

The electrophoretic patterns (fig. 12a and b) show a band in the conalbumin region in all except the first two pools (15 and 20). However, in the double diffusion plates an antigen showing a reaction of identity with the conalbumin line in the egg-white control does not appear until pool 47 (fig. 14). Therefore pools 15 to 41 do not contain conalbumin itself but another unidentified antigen which moves in a similar way to conalbumin on electrophoresis.

This degree of separation, though incomplete, was encouraging. It seemed possible that further treatment of the ovalbumin containing peaks by chromatography might produce a very pure antigen.


Fig.13. Electrophoresis on cellulose acetate paper of whole egg-white and pool 26 of egg-white protein eluted from CM-cellulose (fig.11). The three ovalbumins and conalbumin can be identified in the eluate pool. Electrophoresis carried out for 5 hours.

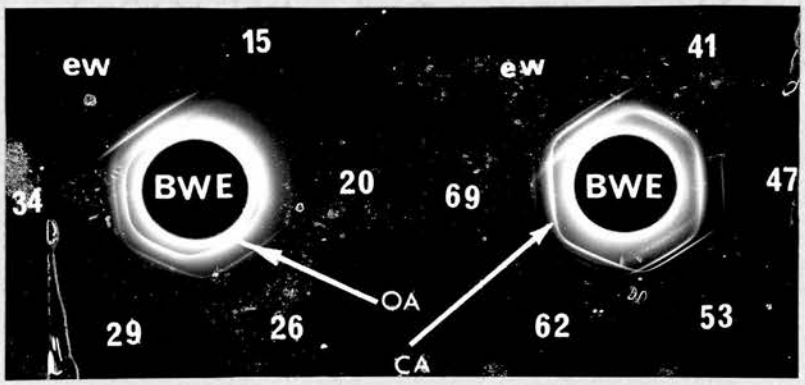



Fig.14. Immunodiffusion analysis of egg-white proteins eluted from carboxymethyl-cellulose by stepwise increases of pH.
The numbers indicate the fraction pools shown in fig.11.
ew = egg-white diluted 1:5.
BWE = anti-egg-white serum.
The arrows point to the ovalbumin and conalbumin precipitation lines.

4. Further purification of the ovalbumin containing portion of the eluate from a primary separation of the egg-white proteins by chromatography on carboxymethyl-cellulose

The fractions constituting the ovalbumin A2 peak from a stepwise elution of egg-white proteins from CM-cellulose were pooled. On electrophoresis (fig. 15) this material was found to contain an antigen running in the conalbumin position, as well as ovalbumin. The pool was dialysed against buffer at pH 4.0 and applied to a CM-cellulose column already equilibrated with the same buffer. The column was then washed through with 200 ml of buffer at pH 4.2.

Elution of the ovalbumins was carried out in a stepwise fashion as described in the Methods section, using the following buffers:

| | |
|---|---|
| 200 ml | pH 4.55 |
| 200 ml | pH 4.70 |
| 200 ml | pH 4.90 |
| 300 ml | pH 6.20 |

The eluate was collected in 20 ml fractions. Their protein content was measured as optical density at 200 mμ in the ultra-violet spectrophotometer, and their pH was checked. The results when plotted (fig. 16) showed mainly a single peak of ovalbumin A2.

Certain fractions were pooled in groups as

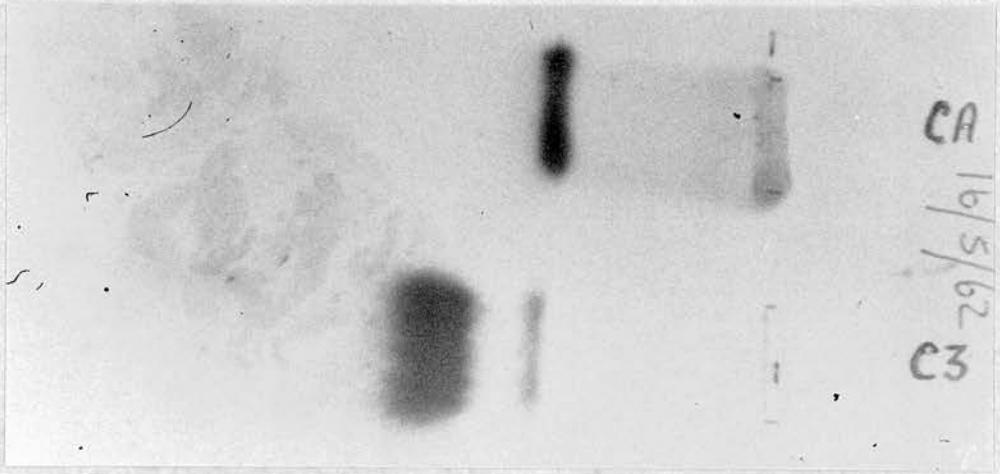



Fig.15. Electrophoresis on cellulose acetate paper.

Above: Electrophoretically pure sample of conalbumin (Dr.I.E.Lush).
Below: Pooled, ovalbumin containing peaks from a primary separation of egg-white proteins by chromatography on CM-cellulose.

Electrophoresis carried out for 2 hours.

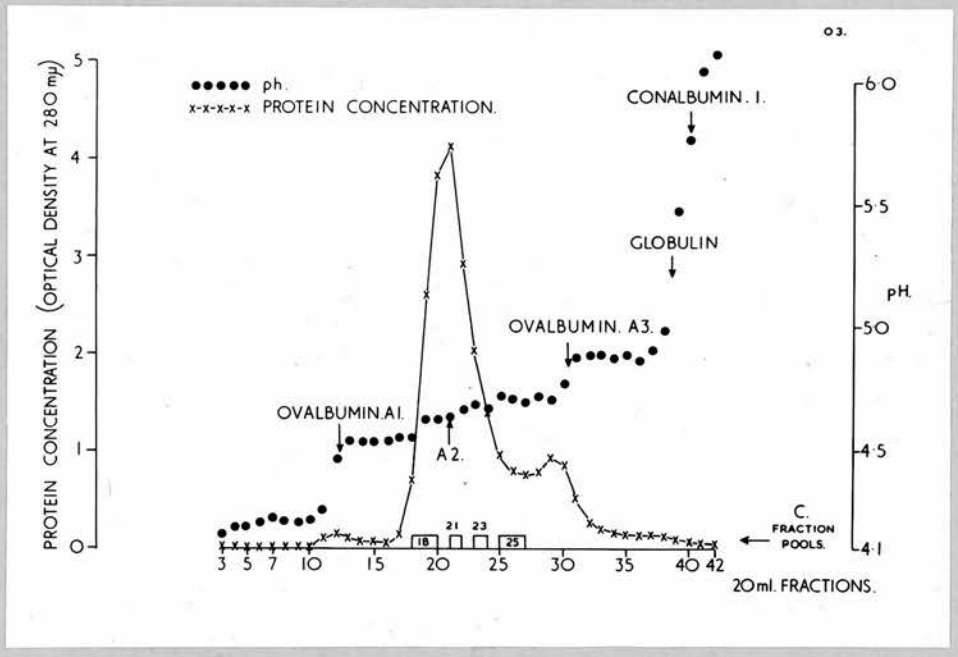



Fig.16. Re-chromatography of the ovalbumin containing peaks from a primary separation of egg-white proteins on CM-cellulose. Elution by stepwise pH changes.

The protein content and pH of each fraction is indicated on the graph. The pH curve is marked at the points given by Rhodes et al. (1958) for the peak elution of each egg-white protein.

shown by the square brackets in figure 16. These were examined to detect the presence of antigens other than ovalbumin.

a) Electrophoresis

No conalbumin or 'globulin' appeared to be present in any of the pools when they were electrophoresed on cellulose acetate paper (fig. 17).

After concentration and freeze drying, one of the pools, number 18, the first part of the main peak, was made up as a 5 per cent solution. The electrophoretic pattern of this alongside that for whole egg-white is shown in figure 18. This ovalbumin concentration is about 4 times that of the ovalbumin in the diluted egg-white (7 per cent in neat egg-white), but no protein other than ovalbumin was detected.

b) Immunodiffusion

In double diffusion against an anti-egg-white serum, the pools showed only ovalbumin. The reactions of two of these (Nos. 18 and 21) constituting the bulk of the peak are shown in figure 6.

With another anti-egg-white serum, one which was particularly sensitive to conalbumin and 'globulin', only one of the four main pools

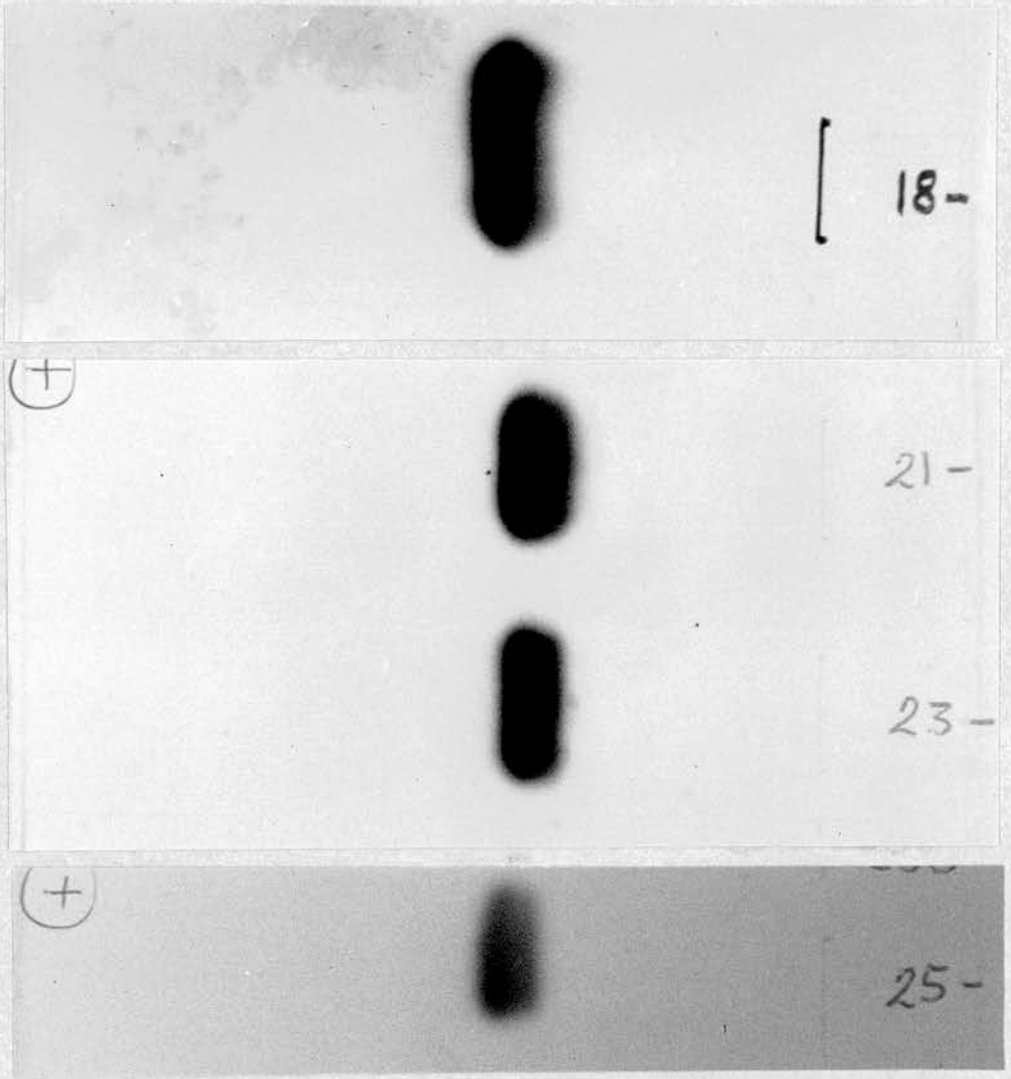



Fig.17. Electrophoresis on cellulose acetate paper of pooled fractions from the elution of ovalbumin from CM-cellulose by stepwise pH changes.
The four pools (numbered as on fig.16) only show protein bands in the ovalbumin position.
Electrophoresis carried out for 2 hours.

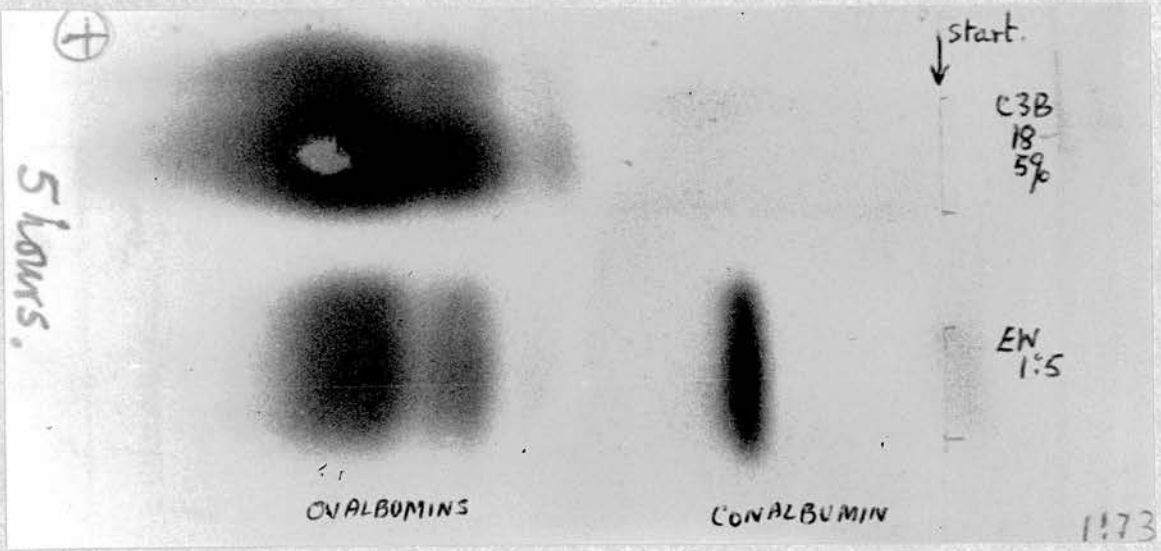



Fig.18. Electrophoresis on cellulose acetate paper.

Above: Fraction pool number 18 (main peak fig.16) from the elution of ovalbumin from CM-cellulose by stepwise pH changes. This material was made up as a 5 per cent solution after freeze drying. This concentration is about four times that of the ovalbumin in the diluted egg-white electrophoresed below.

Below: Whole egg-white diluted 1:5.

Electrophoresis carried out for 5 hours.

(C25) showed signs of the presence of an antigen other than ovalbumin (fig. 19).

When examined with a serum raised against one of the pools (C18) only single lines were produced (fig. 20).

c) Immunoelectrophoresis

In immunoelectrophoresis, developed with an anti-egg-white serum, no antigen other than ovalbumin could be detected in pools 18 and 21 (fig. 21).

These results were evidence that three of the fraction pools contained only one antigen, ovalbumin. No other materials could be detected in them. The results also indicated that the antigen so prepared could safely be used for immunization purposes as only one antibody was seen in sera raised against it.

Of particular importance was the failure to detect any conalbumin or conalbumin-like materials in these preparations. They therefore appeared to be suitable for use as reagents in investigations of the influence of conalbumin on the tanned red cell agglutination test.

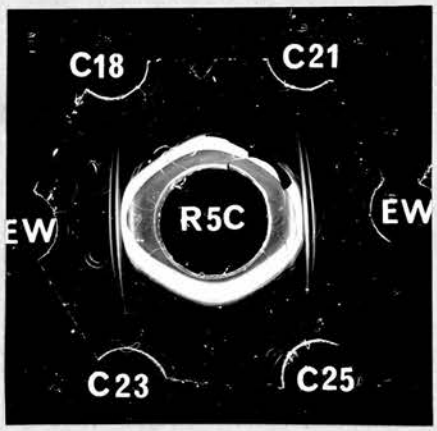



Fig.19. Immunodiffusion analysis in P.B.S. agar of the eluate pools from the re-fractionation on CM-cellulose of partially purified ovalbumin.
C18-C25 = successive fraction pools from the ovalbumin peak shown in fig.16.
EW = whole egg-white diluted 1:5.
R5C = anti-egg-white serum.
On the original plate pool C25 shows traces of another antigen as well as ovalbumin.

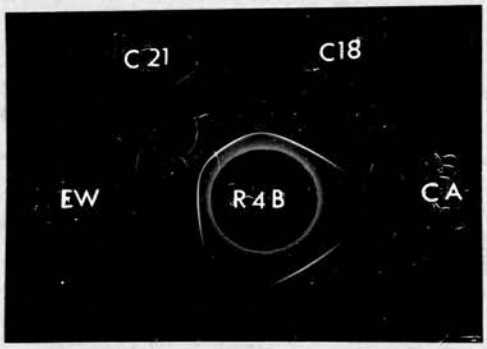



Fig.20. Immunodiffusion analysis in P.B.S. agar.
C18 & C21 = fraction pools from the ovalbumin peak of the re-fractionation of partially purified ovalbumin.
EW = whole egg-white diluted 1:5.
CA = electrophoretically pure conalbumin (Dr.Lush).
R4B = antiserum to fraction pool C18, i.e. anti-ovalbumin.
The only precipitation lines seen are those of the ovalbumin system.

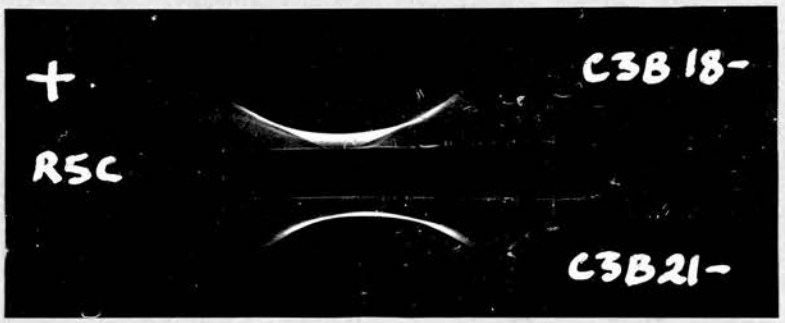



Fig.21. Immunoelectrophoresis of two eluate pools from the re-fractionation of partially purified ovalbumin on CM-cellulose. Developed with an anti-egg-white serum.

C3B18 = pool C18 in fig.16.
C3B21 = pool C21 in fig.16.
R5C = anti-egg-white serum in the centre trough.

5. Further purification of the conalbumin containing portion of the eluate from a primary fractionation of egg-white on CM-cellulose

Considerable success in purifying ovalbumin was obtained by first separating it from egg-white by chromatography on CM-cellulose and then removing residual contaminants by passing it through the column again. It seemed probable that very pure conalbumin could be extracted from egg-white by the same process.

Pooled fractions from the conalbumin containing peaks of a stepwise fractionation of whole egg-white were dialysed against buffer at pH 5.0. They were then applied to a CM-cellulose column which had previously been prepared and equilibrated to pH 5.0 as detailed in the Methods section.

Proteins were eluted from the column by the stepwise sequence of buffers:

| | |
|---|---|
| 200 ml | pH 5.6 |
| 100 ml | pH 5.7 |
| 100 ml | pH 5.8 |
| 200 ml | pH 6.0 |

followed by 0.1 M ammonium acetate.

Fraction collection was begun after the first 150 ml had passed through the column.

The protein content of the fractions was

measured in an ultra-violet spectrophotometer and recorded as optical density at 280 mμ (fig. 22). The pH of each fraction is also shown in the same figure.

This graph shows that there are two marked peaks at points corresponding to the data given by Rhodes et al. (1958) for the elution of conalbumin from CM-cellulose. Certain of the fractions, indicated by the square brackets at the bottom of the graph, were pooled and then examined for purity.

a) Electrophoresis

Cellulose acetate paper electrophoresis showed only a single component in each of the pools examined (fig. 23). In each case this ran in the conalbumin position. The first peak, assumed to be 'globulin', was seen in the same position as the two conalbumins.

b) Immunodiffusion

Figure 24 shows individual (i.e. not pooled) fractions set up in double diffusion against an anti-egg-white serum. The lines are split in some cases but they all appear to be continuous with the line given by a known pure sample of conalbumin on the right-hand plate. Fraction 3 from the 'globulin' peak shows no difference

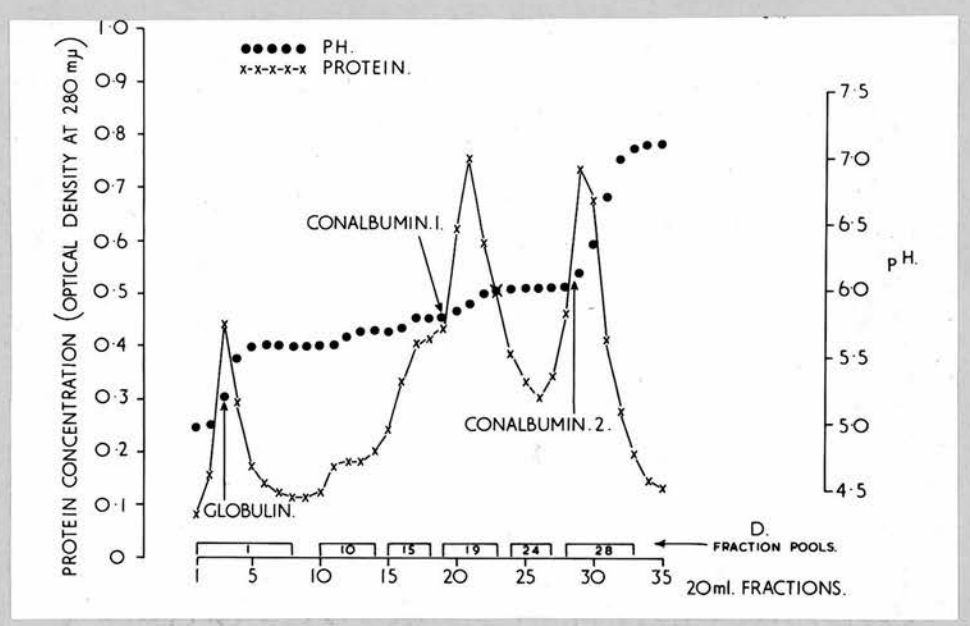



Fig.22. Re-chromatography of the conalbumin containing peaks from a primary separation of egg-white proteins on CM-cellulose. Elution by stepwise pH changes. The protein content and pH of each fraction is indicated on the graph. The pH curve is marked at the points of maximum elution for each of the proteins as reported by Rhodes et al. (1958).

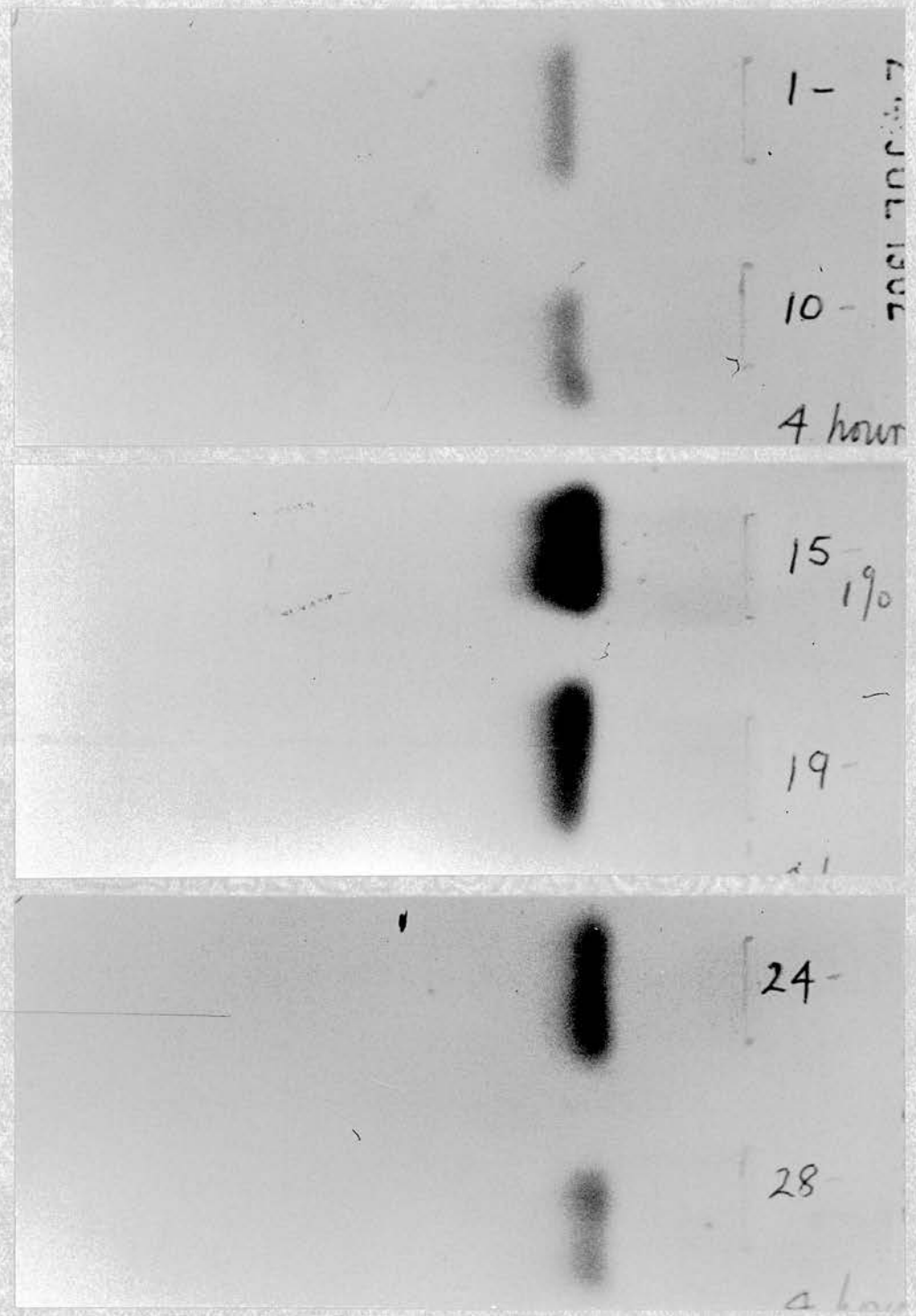



Fig.23. Electrophoresis on cellulose acetate paper of pooled fractions (as shown in fig.22) from the re-chromatography of conalbumin on CM-cellulose. Pool 1 is from the 'globulin' peak. The other pools are from the conalbumin peaks. All the bands are in the conalbumin position.
Electrophoresis carried out for 4 hours.

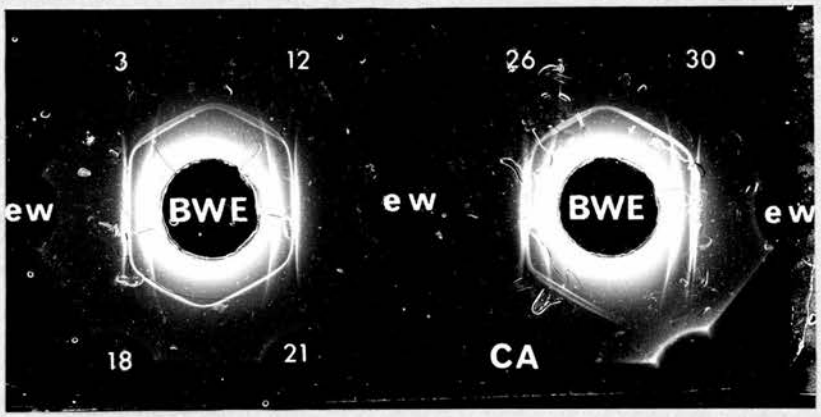



Fig.24. Immunodiffusion analysis of individual fractions from the re-chromatography of conalbumin on CM-cellulose.
3 = fraction from the 'globulin' peak, fig.22.
12-30 = fractions from the conalbumin peaks, fig.22.
ew = whole egg-white diluted 1:5.
CA = electrophoretically pure sample of conalbumin (Dr.I.E.Lush).
BWE = anti-egg-white serum.

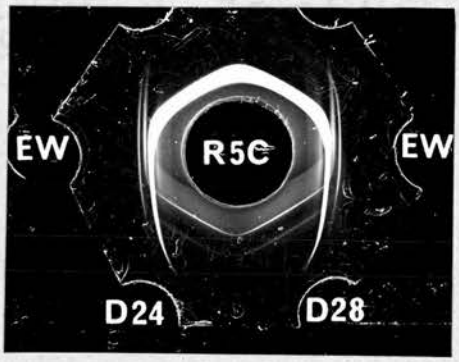



Fig.25. Immunodiffusion analysis of two fraction pools from the re-chromatography of conalbumin on CM-cellulose.
D24 and D28 = pooled fractions from the conalbumin peaks, fig.22.
EW = whole egg-white diluted 1:5.
R5C = anti-egg-white serum.
Lines corresponding to several antigen-antibody systems can be seen with both pools.

from the others.

Two of the pooled groups of fractions were also examined with another anti-egg-white serum (fig. 25). In this case both were seen to contain two well marked components and possibly some ovalbumin as well.

c) Immunoelectrophoresis

Immunoelectrophoresis of pooled fractions when developed with anti-egg-white serum did not show up any antigens other than conalbumin except perhaps in pool 1 from the 'globulin' peak. The patterns for pools 1, 10, 15 and 19 are shown in figure 26. The other two pools gave a similar picture.

These results were disappointing. None of the conalbumin preparations were as pure as had been hoped. They were, however, judged to be sufficiently free of ovalbumin to be useful for investigation of the tanned cell test.

## 6. The separation of egg-white proteins by elution from a CM-cellulose column using a gradient buffer

Crisper separation of the materials eluted from an ion exchange chromatography column is reported to occur if gradient rather than step-

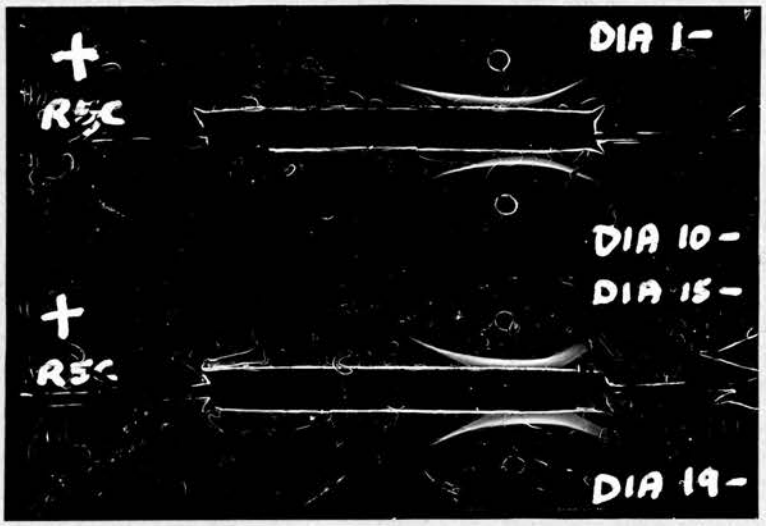



Fig.26. Immunoelectrophoresis of fraction pools from the rechromatography of conalbumin.

D1A1- = D1 'globulin' peak, fig.22.
D1A10-
D1A15-
and D1A19- = D10, D15, and D19 fraction pools from the conalbumin peaks, fig.22.
R5C = anti-egg-white serum in the centre trough.

wise changes in the eluting buffer are used (Kabat and Mayer, 1961). This method of eluting the egg-white proteins from CM-cellulose was therefore tried in the hope that a single pass of the column might be sufficient to produce useful quantities of very pure ovalbumin.

Egg-white was homogenized, dialysed and centrifuged as described in the Methods section, and 191 ml applied to a CM-cellulose column previously equilibrated to pH 4.4. Ovomucoid and ovoflavine were then removed by passing 500 ml of buffer at pH 4.4 through the column.

The remaining proteins were then eluted with a continuously rising pH gradient. The mixing chamber (B in fig. 2) contained 300 ml of buffer at pH 4.4 at the start. The following buffers were then run in successively in 300 ml quantities to raise the pH:

pH 4.7

5.0

5.3

5.6

6.0

6.5

7.0

7.5

The graph in figure 27 shows the pH of each

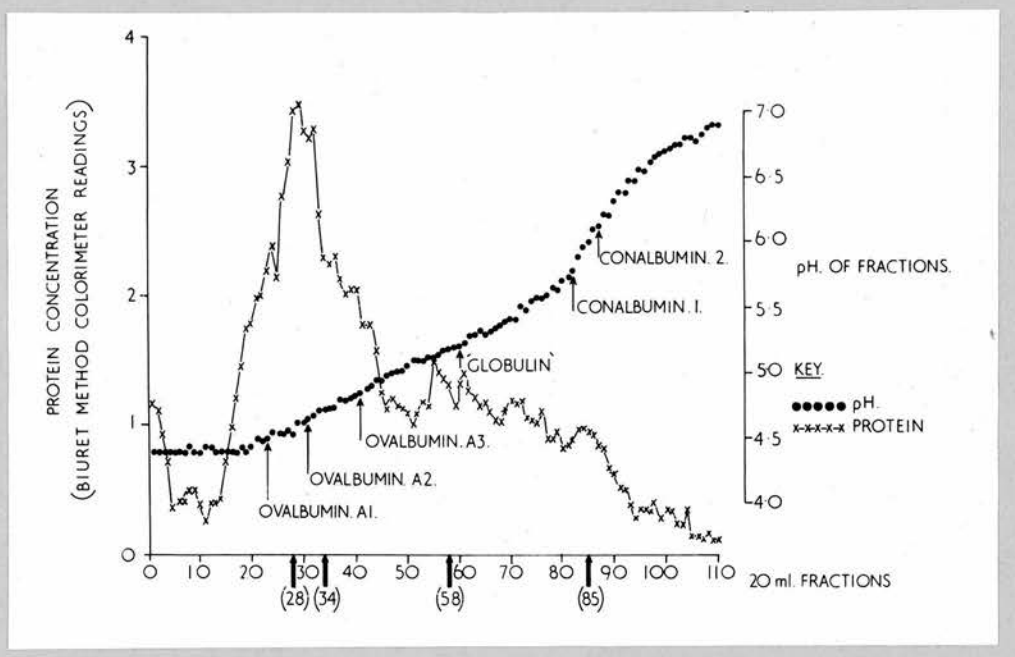



Fig.27. Separation of egg-white proteins by ion exchange chromatography on CM-cellulose. Elution of the proteins by gradient pH change. The protein content and pH of each fraction is plotted on the graph. The pH curve is marked at the points given by Rhodes et al. (1958) for the peak elution of each protein. The fractions examined by immunodiffusion (fig.28) are marked with arrows.

20 ml fraction collected and its protein content as measured by the biuret reaction. The pH curve is marked at the points found by Rhodes et al. (1958) to correspond with the peak elution of each protein.

Four fractions: numbers 28 and 34 from the ovalbumin peak, number 58 from the 'globulin' peak and number 85 from the conalbumin region, were examined by double diffusion against an anti-egg-white serum. The results (fig. 28) showed that the ovalbumin fractions were not particularly pure and that each of the other peaks contained a mixture of all the proteins.

A gradient buffer thus appeared to be less useful than stepwise pH changes for eluting the protein. The latter method was easier to arrange and it also showed promise of being modified to a simple technique useful for the routine preparation of conalbumin-free ovalbumin.

## 7. Development of the routine method used to prepare ovalbumin for use as an antigen

Considerable quantities of ovalbumin were required for the experiments with adjuvants and tanned red cells. The method eventually adopted to separate it from the other egg-white proteins was a combination of crystallization and

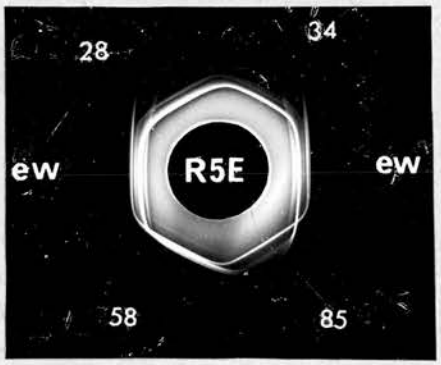



Fig.28. Immunodiffusion analysis in P.B.S. agar of fractions from the elution of egg-white proteins from CM-cellulose by means of a gradient buffer.

28 - 85 = individual fractions from the ovalbumin, 'globulin' and conalbumin peaks as indicated in fig.27.
ew = egg-white diluted 1:5.
R5E = anti-egg-white serum.

chromatography. Crystallization, if carefully carried out, produced quite a pure material though it still contained some conalbumin and other antigens. These were removed by chromatography on CM-cellulose using a simplified technique.

The preliminary purification was carried out by an adaptation of the crystallization methods of Sørensen (1915) and Cole (1933, as described by Koch and Hanke, 1953) using ammonium sulphate. This normally takes 13 days but it was found that even if the time was shortened, as described in the Methods section, a satisfactory product was obtained. On electrophoresis only a faint trace of conalbumin was found to be present in this (fig. 29), much less than that in the commercial product (fig. 10).

The once recrystallized material was further refined by ion-exchange chromatography on carboxymethyl-cellulose. After dialysis against tap water to remove ammonium sulphate, followed by equilibration with the starting buffer at pH 4.2, it was applied to the column. The ovalbumin then appeared as a white band in the upper half. Washing with a pH 4.4 buffer did not move this band.

On eluting the ovalbumin with a buffer of

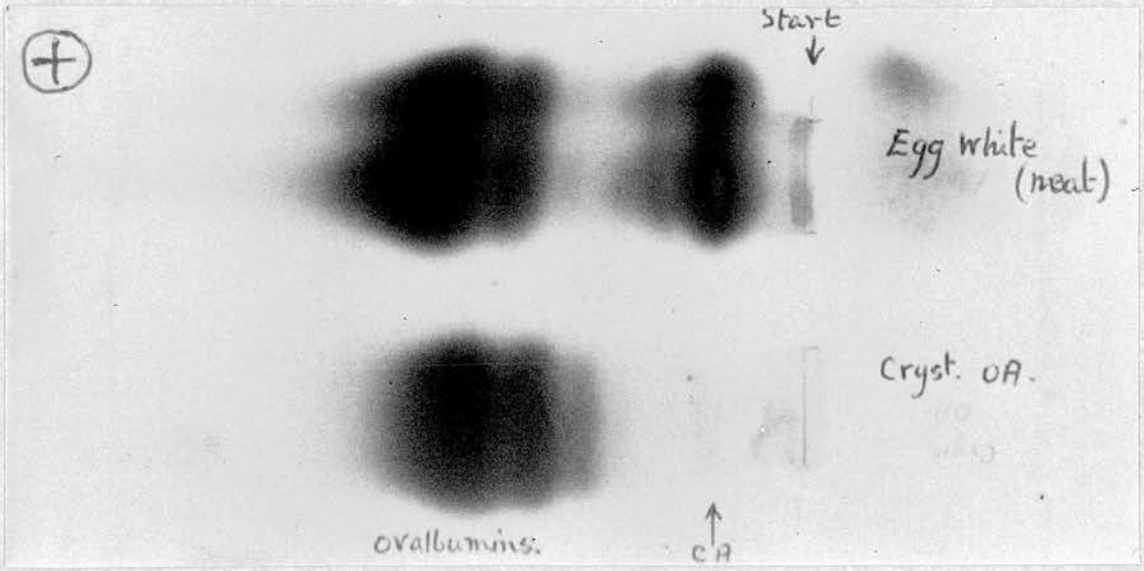



Fig.29. Electrophoresis on cellulose acetate paper.

Above: Neat egg-white.
Below: Once recrystallized ovalbumin prepared by the short method described in this thesis. Note that there is only a very faint trace of conalbumin compared with the large amount present in the commercial sample of twice recrystallized ovalbumin, fig.10.

Electrophoresis carried out for 3 hours.

pH 4.70 the band was seen to move down the column and could be collected as a single fraction probably containing ovalbumins A1 and A2.

A preliminary double diffusion test was set up with this fraction against an anti-egg-white serum. Whilst this was developing, the fraction was reduced in volume with commercial grade CM-cellulose and after that the remaining buffer salts were dialysed away with distilled water.

After freeze drying, a further double diffusion test was carried out to test the purity of the ovalbumin. Figure 30 shows examples of these for four separate batches of ovalbumin prepared by this method. The yield from 100 ml of egg-white was up to 1.1g of ovalbumin.

Ovalbumin separated from egg-white by this method was used for all the experiments reported in the tanned cell and adjuvant sections of this thesis. Complete details of the method of preparation are given in the Methods section.

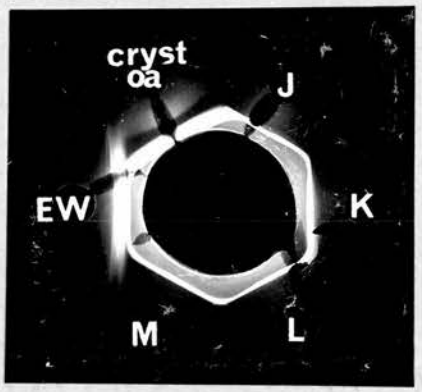



Fig.30. Immunodiffusion analysis of crystallized and chromatographically prepared ovalbumins.

Cryst OA = once recrystallized ovalbumin prepared by the short method described in this thesis.
J,K,L,M, = batches of ovalbumin further purified by chromatography on CM-cellulose after preliminary extraction from egg-white by crystallization.
EW = egg-white diluted 1:20.

The centre well contained anti-egg-white serum R5F.

## C. DISCUSSION AND CONCLUSIONS

The object of this preliminary investigation was to find out whether ovalbumin, which was free of conalbumin by immunological criteria, could be separated from the other egg-white proteins by ion-exchange chromatography.

Experiments with the mineral oil adjuvants and tanned cells would require large amounts of antigen for immunizations and for coating the cells. If ovalbumin prepared by chromatography was as pure as hoped, it would then be necessary to devise a simple, practical and easily reproducible method for carrying out the process. It was essential that the method chosen should not require such continuous and detailed supervision as would interfere with the other investigations being carried forward.

The results of Rhodes, Azari and Feeney (1958) showed that ion-exchange chromatography on CM-cellulose gave sharp peaks for the elution of each of the main egg-white proteins. They were able to remove the three different types of ovalbumin (A1, A2, and A3) separately from the column and they reported them to be conalbumin-free. The ovalbumins were eluted in three well defined peaks over the very small pH range of

0.35 units. A much larger change than this, of 0.95 units, was then required before conalbumin appeared in the eluate. This large pH gap appeared to be a favourable indication that the ovalbumin might really be conalbumin-free.

Rhodes et al. identified each peak by a consideration of the known isoelectric points of the proteins, by special analytic techniques where this was possible, and by comparing them with the same materials prepared by other means. The comparisons were carried out by moving boundary and filter paper electrophoresis which would also give some indication of the purity of the substance eluted in each peak. Rhodes et al. were quite satisfied on this point and of course the very sharpness of the elution peaks was indicative of a purity sufficient for many biochemical purposes.

The present work confirmed the findings of Rhodes et al. and it was also possible to identify some of the eluted proteins by immunological means. Despite the sharp peaks given by stepwise elution of the proteins of egg-white (fig. 11), electrophoresis on cellulose acetate paper showed that an impurity running in the conalbumin position was present in all except the first ovalbumin (A1) peak.

However, this impurity was not conalbumin. None of the precipitation lines produced in a double diffusion test (of the ovalbumin containing eluates against an anti-egg-white serum) showed a reaction of identity with the conalbumin system in egg-white (fig. 14). The first traces of conalbumin were seen in the 'globulin' peak. This was eluted as the pH of the buffer was raised after all the ovalbumin had been removed from the column. The other antigen eluted as a contaminant in the ovalbumin peaks is presumably this 'globulin' material.

No attempt was made to identify 'globulin' specifically but there is some evidence to indicate that it is not a conalbumin. (i) In immunodiffusion tests it gave no reaction of identity with conalbumin. (ii) The line which represented 'globulin' in immunodiffusion tests on the column eluates was seen at its maximum strength in the 'globulin' peak (fig. 14). It was further from the antibody well (showing that it was present at a lower concentration) in the fractions on both sides of the 'globulin' peak. It was therefore a substance eluted at this point and not an early eluate of conalbumin. (iii) The eluate containing the 'globulin' peak did not show the characteristic pink colour of conalbumin

when freeze dried. Conalbumin is an iron binding protein related to the transferrin of serum. It is normally colourless but turns pink with iron. This was apparently picked up from impurities in the commercial grade CM-cellulose used to reduce the volume of the eluates before freeze drying and the freeze dried conalbumin peaks were coloured pink.

It is therefore evident that the straightforward fractionation of egg-white on CM-cellulose by stepwise pH changes is an effective method of preparing conalbumin-free ovalbumin. Unfortunately the ovalbumin is still associated with 'globulin'. This might well interfere with the tanned red cell agglutination test for ovalbumin antibodies in the same manner as conalbumin was said to do (Borduas and Grabar, 1953).

For most other experimental work it would also be preferable to use an antigen which was as pure as possible. It is useful to know that the main substance and not just some impurity has been labelled if antigens are to be traced in the body. Adjuvants may raise unexpectedly high titres to traces of an impurity, leading to confusion. Denaturation, polymerization, adsorption of antigen to an adjuvant or other material are also more surely followed when the

substance used is well characterized. Consideration was therefore given to ways of reducing or eliminating the 'globulin'.

On immunological analysis only the first (A1) peak eluted from CM-cellulose seemed to be relatively free of 'globulin' but the bulk of the eluted ovalbumin was contained in the second (A2) peak (fig. 11). The ovalbumin in fresh egg-white is said to be mainly of type A1 (MacPherson et al., 1944) and it is unlikely that this was not the case with the egg-white applied to the column for the present work. It may be that in the conditions employed some of the ovalbumin changes to the A2 form by the loss of a phosphorous atom. It is, however, more probable that, due to the closeness of the pH of the buffers used for washing to that of those used for elution, much of the A1 type of ovalbumin was lost before elution proper commenced. The analysis carried out by Rhodes et al. did show a high ovalbumin A1 peak but as they were interested in a complete analysis of the egg-white proteins elution was started at a lower pH than that used here.

For the later investigations, ovalbumin was required in some quantity. More could have been obtained if the A1 peak was conserved by carrying

out the preliminary washing with buffer the pH of which was further from the elution point. This, however, might have left the ovalbumin contaminated with ovomucoid and the flavoproteins. The A2 peak therefore seemed to be a useful choice for further treatment to obtain a good yield of very pure ovalbumin.

The method of elution by stepwise buffer changes was, but for the actual composition of the impurity, no great improvement on the crystallization process. When the A1 and A2 peaks were pooled, the electrophoretic pattern given by them was indistinguishable from that of twice recrystallized ovalbumin (compare fig. 10 with fig. 15). However, a single refractionation on CM-cellulose was sufficient to remove this impurity and gave a final product which showed only ovalbumin when tested by the methods and reagents available (figs. 17 and 19).

On the other hand, re-passage of the conalbumin containing fractions through the column, gave materials which were very homogeneous on electrophoresis (fig. 23) but which were shown to be mixtures of many antigens by immunological analysis (fig. 25). This was very disappointing as Rhodes et al. had reported that their conalbumin fractions were very pure. They had, of

course, only tested them by electrophoretic and chemical means.

For the preparation of ovalbumin in quantity a two-stage process appeared to be necessary. Consideration was therefore given to carrying out the primary fractionation of the egg-white by eluting it from the CM-cellulose with a continuously changing gradient buffer arrangement. With the stepwise method, the pH of each washing and eluting buffer had to be very accurately adjusted. The pH meter readings were occasionally erratic, being particularly affected by the operation of thermostats in neighbouring water-baths and incubators. Frequent checking with the standard buffers was necessary. A gradient buffer seemed an easy answer to this as all the wanted pH values would be passed through in sequence. The method was also reported to give better separations (Kabat and Mayer, 1961).

When tried, the separation of the egg-white proteins by this method was found to be very poor (fig. 27). The great width of the column used and hence uneven flow patterns may be one reason for this. When the stepwise elution technique was used each eluate had the opportunity to get well away before the next came off. When a

gradient buffer was used, different elutions might take place in nearby parts of the column at the same time and these might easily become mixed. Another factor may have been overloading of the column though the white appearance which indicated adsorbed protein came only one third of the way down. Rhodes et al. also reported that an increase in column length did not improve the separations obtained with this type of exchanger.

The method of double fractionation by stepwise buffer changes had produced sufficient ovalbumin of great purity to use in the tests on the influence of conalbumin in the tanned cell agglutination test. Chromatography of whole egg-white by this technique was too complicated and time-consuming to be used as a routine method. Consideration was therefore given to carrying out the preliminary separation by the classical method of crystallization. As pointed out above, primary separation of ovalbumin on a column still left as much impurity in the ovalbumin as was found in the crystallized product. The use of chromatography as one stage was, however, important as this would eliminate conalbumin.

Two substances cannot be cleanly separated

by crystallization. Each step increases the difference between the amount of each substance present but does not eliminate one of them. Many times repeated recrystallization of ovalbumin might result in a very pure product but by definition it would still contain traces of most of the other antigens of egg-white.

An additional difficulty is that the crystallization process is very dependent on the physical conditions under which it is carried out. A stable temperature for each of the steps is important. This is not at all easy to achieve day and night in an ordinary laboratory or during centrifugations, etc.

Chromatography, on the other hand, is very much a one-step process in which the ionic strength and pH of the materials can be rigidly controlled. It can be done over a relatively short time with everything at one temperature for the few hours work involved. In addition, it would be bad luck if the two processes were not complementary in that each would reduce some impurity more effectively than the other and so have an overall additive effect.

The crystallization method described in the Methods section, though only a rather coarse copy of the standard technique, did produce ovalbumin

which was much purer than that bought in from a commercial source (fig. 29). When followed by simple chromatography, the remaining impurities were reduced to undetectable limits with the minimum of difficulty and complication. It is not suggested that the ovalbumin was absolutely pure, hyperimmunization of test animals can show up faint traces of contaminating antigens in any material; only the use of artificial polypeptides can be considered to fulfil a need for absolute purity (Maurer, 1963).

The method described of employing crystallization followed by chromatography does, however, appear to be a simple way of producing very pure ovalbumin for immunological investigations.

# Part Four

## Experimental

## THE TANNED RED CELL AGGLUTINATION TEST

# EXPERIMENTAL

## THE TANNED RED CELL AGGLUTINATION TEST



## A. INTRODUCTION

The use of tanned red cells coated with antigen to detect small quantities of antibody by

their agglutination was first described by Boyden in 1951. Stavitsky (1954a) developed and popularized the technique and it is now one of the most sensitive and convenient of the methods available for titrating serum antibodies. So reliable has it become that packaged tests, for thyroid auto-antibodies and for pregnancy, which employ the technique, are now marketed.

Difficulties still confront the laboratory worker using the test under rigorous experimental conditions. Chief amongst these are: the short time which the cells can be kept after preparation, the great variation in sensitivity found between different batches, and the problem of deciding exactly which antigen is reactive when the cells are coated with mixed materials.

Optimum storage conditions for fresh tanned and coated red cells would require them to be sterile but this is difficult to achieve in view of the repeated treatments and washings required during their preparation. Most authors prepare the cells freshly for each set of titrations and carry these out on one day (i.e. George and Vaughan, 1962). With some antigens, such as thyroglobulin, it is possible to preserve the cells after coating by treating them with formalin (Fulthorpe et al., 1961). The

resultant cells show little loss of sensitivity.

Other antigens are damaged by the formalin and the preserved cells are non-reactive (Fulthorpe, 1962). To avoid this some authors have preserved the cells first and then tanned and coated them (McKenna, 1957; Ingraham, 1958; Fahey and Humphrey, 1962). McKenna reported that titrations carried out with his cells showed good agreement with the results from freshly prepared material. They could also be frozen or lyophilized without damage. Fahey and Humphrey (1962) formalinized their cells by the method of Csizmas (1960). This is simple and quick to carry out and the preserved cells are unagglutinated and undistorted, two faults which complicate other methods.

Preservation of the sensitized cells in large batches is one way round the great variation shown by different lots of cells coated with a single stock of antigen (Dutton et al., 1960). A number of investigations into the reasons for this variation have been carried out. Boyden (1951), using soluble protein antigens, and Felton et al. (1961), working with herpes simplex virus, determined the optimal concentration of tannic acid, pH at coating, and the time of exposure to the coating material, by

empirical experiments. Steffen and Rosak (1963) investigated the effect of varying the concentration of antigen. George and Vaughan (1962) followed up Dutton et al.'s (1960) observation that turbid solutions of ovalbumin were better coating materials than clear ones, by investigating the effect on the test's sensitivity of urea denaturation of the antigen. All these investigations were done with fresh, unpreserved, cells.

The third problem confronting the user of tanned cells, is to determine to what antigen they are reacting when the coating material is at all impure. Borduas and Grabar (1953) were the first to report that the contamination of ovalbumin coated cells with another egg-white protein conalbumin, may cause them to give misleading results. In their view, conalbumin was much more readily attached to the cells than was ovalbumin.

Vaughan and Kabat (1953) also pointed out the dangers of this combination of antigens, but Stavitsky (1954b) did not think it important as long as ovalbumin which had been recrystallized many times was used for coating. In this he was supported by George and Vaughan (1962).

Linz and Lecocq (1962) and Lecocq and Linz (1962) investigated the effect of coating tanned

cells with several antigens, either successively or mixed together. They found that some antigens such as horse, bovine, or rabbit whole serum appeared to be 'strong' in that they played the predominant role when used with 'weak' antigens such as bovine or human serum albumin or egg-white. As the 'strong' antigens were themselves complex mixtures, the picture is still in rather a confused state.

In the present investigation experiments were therefore carried out to determine the optimum conditions for coating formalinized tanned cells and the influence of traces of conalbumin on the specificity of titres of anti-ovalbumin recorded by the test.

## B. RESULTS

### 1. Examination of the antigens chosen for use in the investigation of the influence of conalbumin on the tanned cell agglutination test.

For use in the experiments on the influence of conalbumin on the tanned red cell agglutination test for anti-ovalbumin antibodies, two chromatographically prepared antigens were chosen. The ovalbumin used was preparation C18 (see fig. 16) and the conalbumin was preparation D28 (see fig. 22).

These materials had already been examined by electrophoresis and immunodiffusion immediately after their elution from the chromatography columns. The ovalbumin appeared to be very pure indeed, but traces of other antigens were present in the conalbumin. These two antigens were now examined further by immunodiffusion and immunoelectrophoresis using different antisera.

For the proposed experiments with tanned cells it was also necessary to prepare sera absorbed by each of the antigens. Precipitin curves were constructed to facilitate this and these also gave additional information as to the purity of the antigens used.

a) Ovalbumin

(i) The material chosen (C18) appeared to be extremely pure when examined after chromatography. A potent serum was raised against it in the rabbit, as detailed in the Methods section. This serum gave only one line in double diffusion against its own antigen and also against whole egg-white (fig. 31). When the serum was used to develop immunoelectrophoretic separations, a faint trace of another antigen showed up with egg-white but only a line corresponding to ovalbumin with its own antigen (fig. 32).

When the ovalbumin was examined in double diffusion with anti-egg-white serum R5E, traces of another antigen other than ovalbumin were seen (fig. 33). This antigen was presumably a remainder of the 'globulin' found in the ovalbumin fractions after primary separation of whole egg-white on CM-cellulose (fig. 14). It showed no reaction of identity with conalbumin. The serum used (R5E) did not give so many lines with whole egg-white or crystalline ovalbumin as did the anti-egg-white serum BWE (fig. 8) though this latter serum detected no impurity in the ovalbumin C18 (fig. 6).

(ii) The material was further examined by

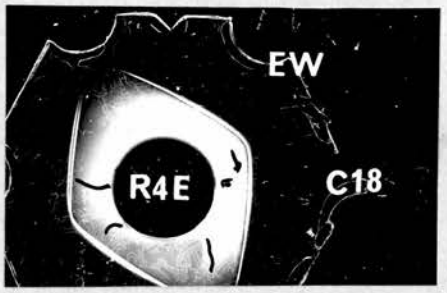



Fig.31. Immunodiffusion analysis of an anti-ovalbumin serum.
R4E = antiserum to ovalbumin preparation C18.
EW = whole egg-white diluted 1:10.
C18 = chromatographically purified ovalbumin.
The plate was prepared with Ion-agar in tris buffer pH 8.65 (Poulik,1957).

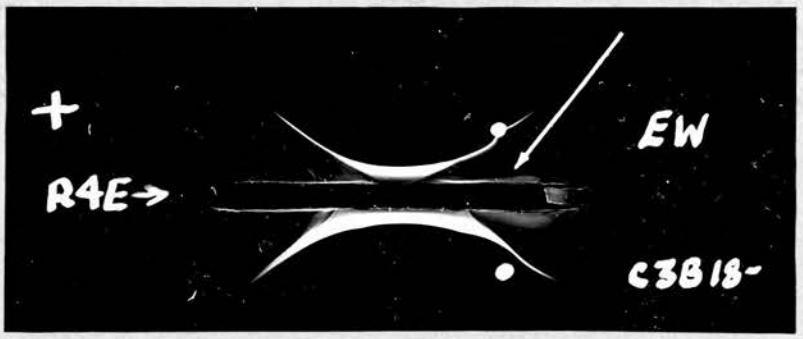



Fig.32. Immunoelectrophoretic analysis of an anti-ovalbumin serum.
EW = whole egg-white.
C3B18 = chromatographically prepared ovalbumin batch C18.
R4E = antiserum raised against ovalbumin preparation C18.
The arrow points to a faint precipitation arc indicating that the serum is reacting to another antigen in egg-white as well as to ovalbumin.


Fig.33. Immunodiffusion analysis of the ovalbumin and conalbumin preparations chosen for use in tests with tanned cells.

R5E = anti-egg-white serum.
C18 = chromatographically purified ovalbumin, 1 per cent solution.
A faint line can be seen towards the antigen well in addition to the chief ovalbumin line. The split in this latter is an artifact.
D28 = Chromatographically purified conalbumin, 5 per cent solution. This is seen to contain a trace of ovalbumin as well as conalbumin and other antigens.
EW = whole egg-white diluted 1:10.
Coml OA = twice recrystallized ovalbumin, commercial source.

This plate was prepared with 1 per cent Ion-agar in tris buffer (Poulik,1957).

constructing curves of its precipitation characteristics with sera which would themselves be used in the later experiments. These were an anti-egg-white serum (R5C) and a serum (R4C) raised against the antigen C18 itself.

The tests were carried out in capillary tubes. The heights of precipitate at each antigen dilution are shown in table 9 and in the graph, figure 34. Sharp peaks of precipitation were produced which suggested that the material was very pure indeed.

Table 9. Height of precipitate produced by the reaction of dilutions of a 1 per cent solution of ovalbumin C18, with equal volumes of antiserum.

| Serum | Dilutions of Antigen | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1:2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 |
| Anti-egg-white R5C | 5.5 | 6.5 | 8.0 | 15.0 | 22.0 | 11.5 | 8.0 | 3.0 | 2.0 | 1.0 mm |
| Anti-ovalbumin R4C | 5.5 | 7.0 | 10.5 | 23.0 | 21.5 | 14.0 | 8.5 | 3.0 | 2.0 | 1.0 mm |

When another anti-egg-white serum became available a precipitation curve was constructed for it also. The results, table 10, are shown graphically in figure 35. The peak of the

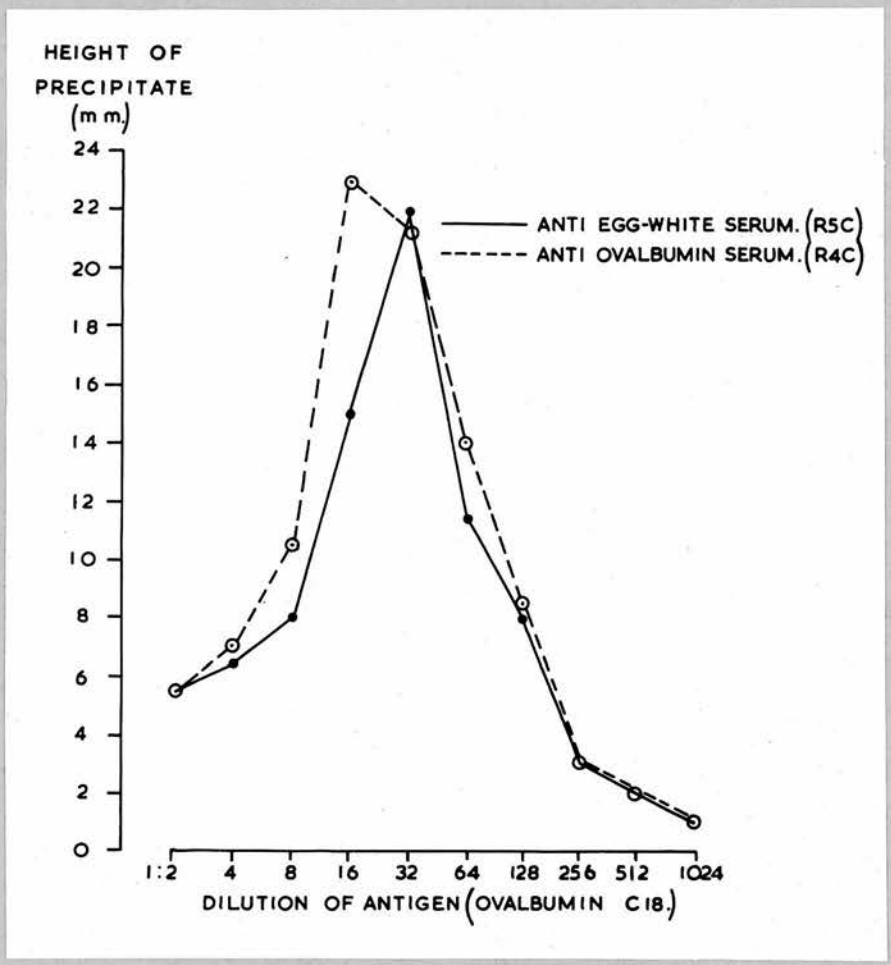



Fig.34. Precipitation curves (capillary tube method) given by anti-ovalbumin and by anti-egg-white sera with chromatographically purified ovalbumin. .

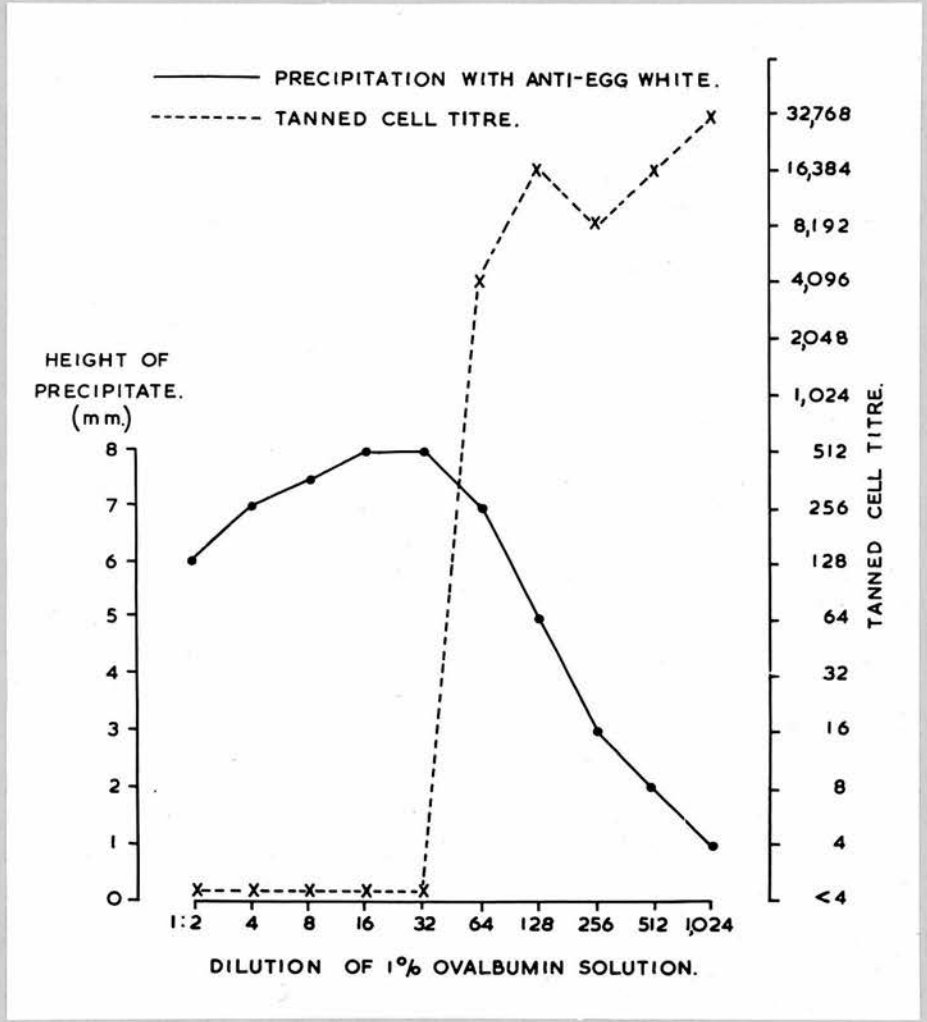



Fig.35. Precipitation curve(capillary tube method) given by anti-egg-white serum R5E with chromatographically purified ovalbumin C18. The titres of unused antibody at each point on the curve were measured with tanned cells coated with ovalbumin and are shown by the broken line.

curve is not as sharp as that in figure 34. This serum (R5E) was that which, in a double diffusion test (fig. 33), had shown up the faint line of another antigen-antibody system with the purified ovalbumin.

Table 10. Height of precipitate produced by the reaction of dilutions of a 1 per cent solution of ovalbumin C18 with equal volumes of neat antiserum.

| Serum | Dilutions of Antigen Solution | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 1:2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 |
| Anti-egg-white R5E | 6.0 | 7.0 | 7.5 | 8.0 | 8.0 | 7.0 | 5.0 | 3.0 | 2.0 | 1.0 mm |

(iii) The relationship of the tanned cell agglutination test to the precipitation curves was also investigated. Portions of the anti-ovalbumin serum R4C and the anti-egg-white serum R5E were absorbed with each of the antigen dilutions used to construct the curve and the antibody then remaining in them was titrated.

Doubling dilutions of a one per cent solution of ovalbumin were made in 0.5 ml volumes of saline in the wells of a W.H.O. plastic plate. From each well 0.2 ml was transferred to a Durham tube and 0.2 ml of neat serum added. The tubes

were then corked, inverted several times to mix the contents, and placed in an incubator at 37° for two hours. They were then left at 4° overnight and in the morning centrifuged at 3000 G for 30 minutes at 4°. The supernate from each was then transferred to a clean Durham tube and titrated with fresh tanned cells coated with ovalbumin.

The titres found for each serum are given in table 11. Curves showing the change in titre at each point have been superimposed on the precipitation curves in figures 35 and 36. In both cases there is a sharp rise in titre as soon as the equivalence point is passed. Removal of agglutinating antibody is therefore complete at the point of peak precipitation as shown by the capillary tube test.

b) Conalbumin

Materials eluted from CM-cellulose in the conalbumin containing peaks were always found to contain more than one antigen even after repeated passages of the column. One of these materials (D28) was chosen and examined further.

It was made up as a 5 per cent solution (the conalbumin concentration in normal whole egg-white is about 0.3 per cent) and set up in double

Table 11. Tanned cell titres of sera absorbed with various dilutions of a 1 per cent solution of ovalbumin C18.

| Dilution of Antigen | | 1:2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Titre of supernates from serum | Anti-OA R4C | <4 | <4 | <4 | <4 | 32 | 2,048 | 8,192 | 65,536 | 32,768 | ND |
| | Anti-EW R5E | <4 | <4 | <4 | <4 | <4 | 4,096 | 16,384 | 8,192 | 16,384 | 32,768 |

ND = not done.

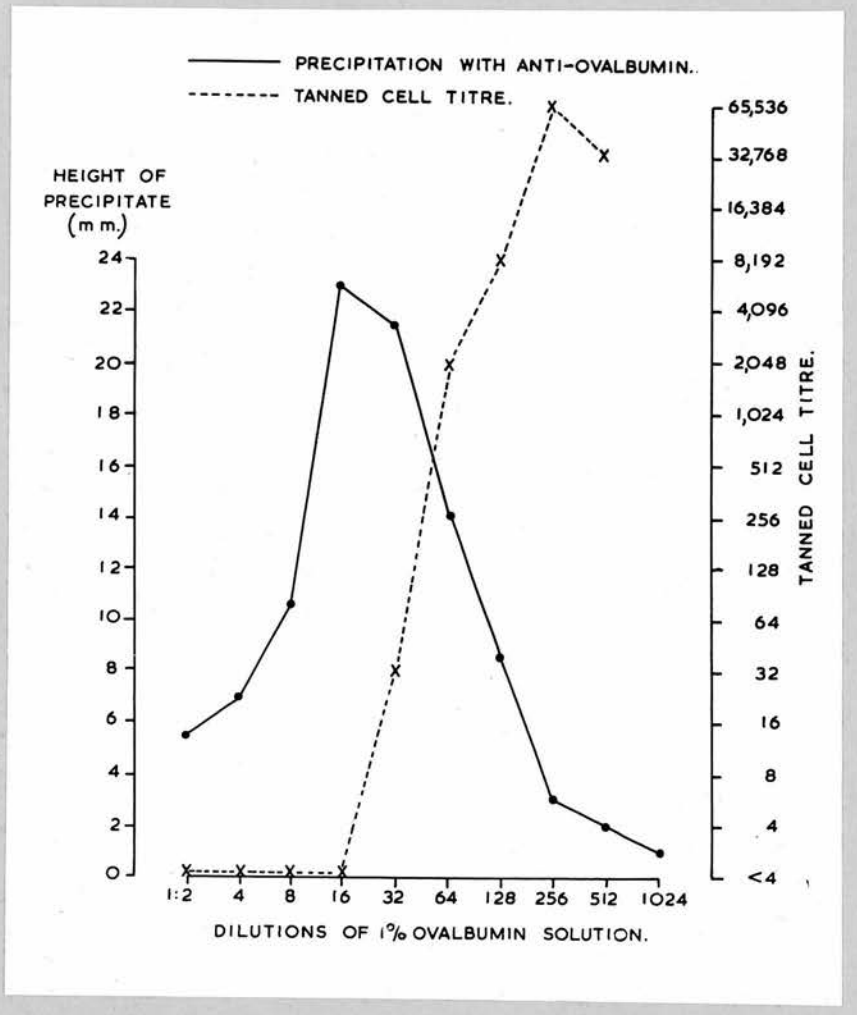



Fig.36. Precipitation curve of anti-ovalbumin serum R4C with chromatographically purified ovalbumin (cf. fig.35).
The titres of unused antibody at each point on the curve were measured by tanned cells coated with ovalbumin and are indicated by the broken line.

diffusion against anti-egg-white serum R5E. The result (fig. 33) showed that at least one protein other than conalbumin was present and that there also appeared to be a trace of ovalbumin in it.

Immunoelectrophoresis showed faint traces of two slow moving components in addition to conalbumin, but no sign of any ovalbumin (fig. 37).

Precipitation curves were prepared for this material against anti-egg-white sera and an anti-ovalbumin serum which were to be used in the later experiments. The results are given in table 12, and are plotted in figure 38. Their flattened shape with anti-egg-white sera is consistent with there being more than one antigen-antibody system present. There is a slight precipitate with the anti-ovalbumin serum in the region of high antigen concentration. This indicates that the antigen contains some ovalbumin rather than that the serum contains any anti-conalbumin antibody.

c) Conclusions

In the characterization of these antigens and of eluates from CM-cellulose columns, classical Ouchterlony double diffusion tests showed themselves to be particularly useful.

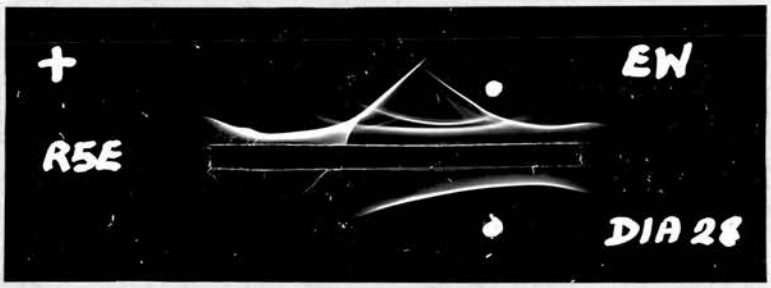



Fig.37. Immunoelectrophoretic analysis of chromatographically prepared conalbumin D28.

R5E = anti-egg-white serum in the centre trough.
EW = whole egg-white.
D1A28 = chromatographically prepared conalbumin D28. Precipitin arcs corresponding to two antigens in addition to conalbumin can be seen. There is no arc in the ovalbumin position.

Table 12. Heights of precipitate produced by the addition of dilutions of 1 per cent conalbumin D28 to equal volumes of neat antiserum.

| Serum | Dilution of the Antigen Solution | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Neat | 1:2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 |
| Anti-egg-white BWE | ND | 3.25 | 3.25 | 3.25 | 3.5 | 3.25 | 3.25 | 3.0 | 2.0 | 1.25 | 0.5 mm |
| Anti-egg-white R5C | ND | 4.0 | 4.0 | ND | 3.5 | 3.5 | 3.0 | 2.25 | 1.25 | 0.75 | 0.25 mm |
| Anti-ovalbumin R4C | 1.0 | 0.5 | 0.25 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 mm |

ND = not done.

0 = no precipitate.

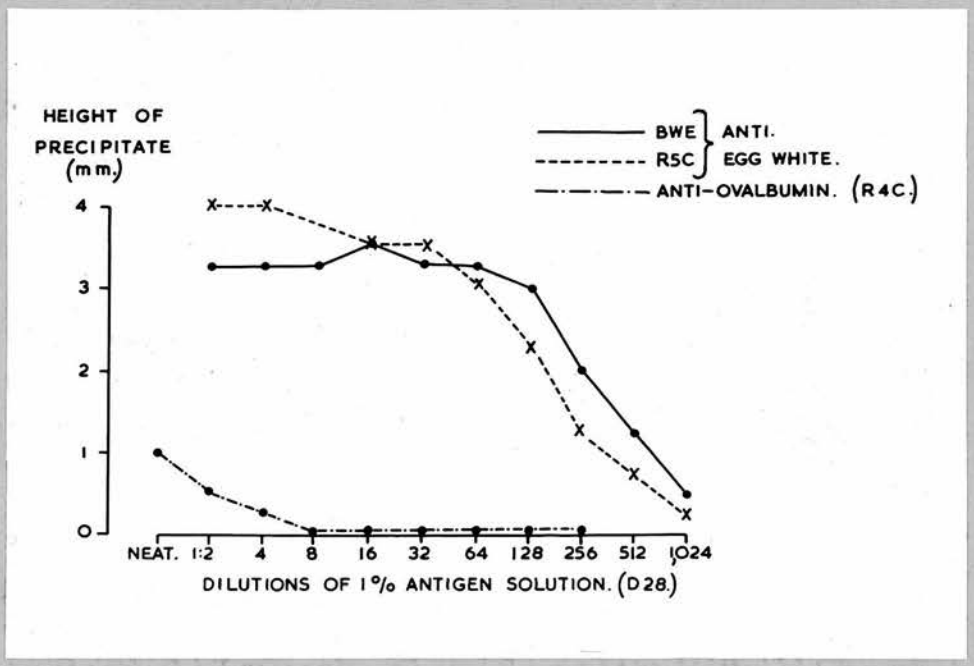



Fig.38. Precipitation curves (capillary tube method) given by anti-egg-white and anti-ovalbumin sera with chromatographically purified conalbumin.

They gave both an analysis of the unknown mixtures of proteins and an immediate identification of some of the components revealed. The use of several different sera helped to improve the resolving power of the method.

Electrophoresis in cellulose acetate paper was only useful as a rather crude guide to the materials present. Immunoelectrophoresis was an advance, but in the author's hands did not reveal more components than did double diffusion. This may have been due to the small size of the samples used.

Hyperimmunization of animals with the antigens was expected to be a potent method of detecting impurities. Antigens present in trace amounts would be expected to stimulate the production of relatively more antibody than their concentration in the mixture might suggest. This would be particularly evident as antibodies to the major antigens reached a plateau after several injections. Antibody to a trace contaminant in ovalbumin was produced in this manner (fig. 32). Its very low level was taken to indicate that the impurity would be of little importance in simple experimental immunizations.

The precipitin tests were interesting in that a very high peaked curve was obtained with

ovalbumin and the earlier rabbit sera (fig. 34). This was of the type which would be expected with H (horse) rather than R (rabbit) type antibodies. A later, more hyperimmune, rabbit serum gave a much more typical curve (fig. 35). In the case of conalbumin, known to contain several antigens, a flattened curve was produced (fig. 38). This was consistent with several antigen-antibody systems with different equivalence points being present at the same time (Cohn et al., 1949). The peaked curves seen with ovalbumin therefore appeared to be the reactions of a single antigen-antibody system.

Titration of supernates from the precipitin tests showed that absorption of sera just on the antigen excess side of the equivalence point would be effective in removing agglutinating antibodies. Sera so absorbed could therefore be used as special reagents in later investigations.

This was also additional evidence for the purity of the ovalbumin. George and Vaughan (1962) found that only when using a chromatographically and electrophoretically purified (but otherwise uncharacterized) preparation of ovalbumin to coat tanned red cells did neutralization of agglutination occur at the equivalence point

of the precipitation curve. With less highly purified ovalbumin there was residual haemagglutination far into the region of antigen excess.

Characterization of the two antigen preparations ovalbumin C18 and conalbumin D28 by the above methods showed that both contained traces of other antigens in addition to the chief one. In the case of ovalbumin the contaminant was only present in a very small quantity. It was not conalbumin and no conalbumin could be detected. The conalbumin preparation, on the other hand, contained traces of several antigens one of which was ovalbumin.

2. To determine whether the addition of conalbumin to the ovalbumin coating of tanned red cells, alters the specificity of their reactions

Three batches of fresh tanned red cells were prepared:

Batch One was coated with chromatographically prepared ovalbumin (C18, see fig. 16).

Batch Two was coated with chromatographically prepared conalbumin (D28, see fig. 22).

Batch Three was coated with a mixture of the two antigens in the ratio, three parts of ovalbumin to one part of conalbumin.

High titred rabbit sera which had been

raised against ovalbumin (both crystalline and chromatographically prepared) and against egg-white, were titrated with the three batches of cells. The sera were also titrated after they had been absorbed with ovalbumin (C18) or conalbumin (D28) and the precipitates removed as described in Methods section 7 . These absorptions were carried out on the antigen excess side of the equivalence points of the sera as determined by capillary tube precipitation tests.

In the case of the sera raised against chromatographically purified ovalbumin, any anti-conalbumin antibodies which might possibly be present were removed by adding an equal volume of 1 per cent conalbumin solution to the serum.

The results of the titrations are presented in tables 13, 14 and 15. Where possible the antibody nitrogen content of each serum antibody was determined and is entered on the tables. Insufficient serum was available for this to be done in all cases.

The results obtained can be analysed as follows:

Table 13. Tests with sera raised against chromatographically purified ovalbumin.

a) When an anti-ovalbumin serum was

absorbed with ovalbumin it showed no titre to cells coated with ovalbumin or to cells with conalbumin in their coating. Therefore, where cells coated with conalbumin recorded a titre for a serum after that serum had been absorbed with ovalbumin the titre recorded must have been a record of anti-conalbumin antibody.

The very small titres remaining after the absorption of serum R4C with ovalbumin are probably slight traces of anti-ovalbumin from this very potent serum. To completely remove all traces of precipitating antibody from a serum it is usually necessary to carry out a second precipitation after removal of the main precipitate.

b) The conalbumin coated cells recorded a good titre of anti-ovalbumin. Absorption of the serum with conalbumin did not affect this or only reduced the titre by a single dilution. Therefore the cells cannot have been responding to anti-conalbumin in the serum. The trace of ovalbumin present in the conalbumin must have been sufficient to fully sensitize these cells.

The only other possibility would be that the anti-ovalbumin serum contained antibody to an impurity ('globulin') with which the conalbumin cells were also coated and that they were

Table 13. Titres of anti-ovalbumin sera.

| Serum | Antibodies present | µg antibody nitrogen/ml | Titre to cells coated with: | | |
|---|---|---|---|---|---|
| | | | Ovalbumin | Conalbumin | Ovalbumin 3 parts Conalbumin 1 part |
| R4B Unabsorbed | Anti OA | 41 | 40,960 | 5,120 | 5,120 |
| R4B Absorbed with conalbumin | Anti OA | 41 | 20,480 | 2,560 | 5,120 |
| R4B Absorbed with ovalbumin | - | - | < 10 | < 20 | < 10 |
| R4C Unabsorbed | Anti OA | ND | 5,242,000 | 327,000 | 655,000 |
| R4C Absorbed with conalbumin | Anti OA | ND | 2,621,000 | 327,000 | 327,000 |
| R4C Absorbed with ovalbumin | - | ND | 40 | 80 | 80 |

ND = not done.

reacting to this. Traces of another antibody were seen in hyperimmune anti-ovalbumin sera (fig. 32) but at an extremely low level. It is therefore unlikely that they could be responsible for the pattern of titres shown here.

Table 14. Tests with a serum raised against twice-recrystallized ovalbumin.

This serum contained about equal amounts of both anti-ovalbumin and anti-conalbumin antibodies.

a) When the serum was absorbed with ovalbumin, all antibody reacting with the ovalbumin coated cells was removed (bottom line). Therefore the titres recorded by conalbumin coated cells must be antibody to conalbumin.

b) Conversely, antibody recorded in the middle line (sera absorbed with conalbumin) must be antibody to ovalbumin.

c) The antibody nitrogen content of the serum for each antibody was similar and the cells, whether coated with conalbumin alone or mixed with ovalbumin, gave similar titres for each antibody. For the whole serum, the titres recorded were slightly higher. This additive effect would be expected where two different antibodies were present in similar quantities.

Table 15. Tests with anti-egg-white sera.

a) Once again, absorption with ovalbumin left the sera unreactive to ovalbumin coated cells. The bottom line in the table for each serum must therefore represent a titre of anti-conalbumin.

b) With each of the sera, the cells coated with the mixed antigens recorded a titre for that serum after one of the antibodies in it had been removed. These titres were in proportion to the actual quantity of antibody present. With serum BWE an additive effect is seen when both antibodies are present in the unabsorbed serum. As would be expected this is not seen with serum R5C which contains a great excess of anti-ovalbumin.

These results show that:

a) Cells which have conalbumin as well as ovalbumin in their coating are not more sensitive to anti-conalbumin than they are to anti-ovalbumin. They give titres which are in proportion to the actual antibody content of the serum. Therefore the titre which they show for an unabsorbed serum is that of the antibody present in the greatest quantity. When the same amount of each antibody is present there may be a slight additive effect.

Table 14. Titres of anti-twice recrystallized ovalbumin (commercial source).

| Serum | Antibodies present | µg antibody nitrogen/ml | Titre to cells coated with: | | |
|---|---|---|---|---|---|
| | | | Ovalbumin | Conalbumin | Ovalbumin 3 parts Conalbumin 1 part |
| BWC Unabsorbed | Anti OA + Anti CA | 16 + 15 = 31 | 20,480 | 20,480 | 10,240 |
| BWC Absorbed with conalbumin | Anti OA | 16 | 10,240 | 5,120 | 5,120 |
| BWC Absorbed with ovalbumin | Anti CA | 15 | 10 | 2,560 | 5,120 |

Table 15. Titres of anti-egg-white sera.

| Serum | Antibodies present | µg antibody nitrogen/ml | Titre to cells coated with: | | |
|---|---|---|---|---|---|
| | | | Ovalbumin | Conalbumin | Ovalbumin 3 parts Conalbumin 1 part |
| BWE Unabsorbed | Anti OA + Anti CA | 38 + 68 = 106 | 327,600 | 327,600 | 655,300 |
| BWE Absorbed with conalbumin | Anti OA | 38 | 163,800 | 81,920 | 327,600 |
| BWE Absorbed with ovalbumin | Anti CA | 68 | < 10 | 81,920 | 327,600 |
| R5C Unabsorbed | Anti OA + Anti CA | 234 + 48 = 282 | 2,621,000 | 327,600 | 655,300 |
| R5C Absorbed with conalbumin | Anti OA | 234 | 1,310,000 | 327,600 | 655,300 |
| R5C Absorbed with ovalbumin | Anti CA | 48 | < 10 | 81,920 | 163,800 |

b) Very small quantities of ovalbumin present as an impurity in a conalbumin preparation are sufficient to sensitize cells to anti-ovalbumin antibodies.

c) Conalbumin appears to be a potent antigen in the rabbit. Antisera to ovalbumin-prepared-by-crystallization may contain an unexpectedly high level of antibody to conalbumin. If unsuspected, these antibodies could cause confusion in passive haemagglutination tests carried out with cells coated with the same crystallized ovalbumin.

3. The preservation of tanned and coated sheep red cells

Fresh sheep red cells, tanned and then coated with antigen constitute a most sensitive test for antibody. Unfortunately they suffer from two disadvantages:

(i) They have to be used immediately after preparation. Only if conditions are favourable may they sometimes be used for three or four days, before bacterial contamination and lysis occurs.

(ii) Each batch of cells prepared may diverge widely from others in its sensitivity to antibody. This variation is a great disadvantage for

measuring antibody levels in experiments lasting several months unless all the sera are examined at the same time.

To overcome these difficulties, the known methods of preserving tanned and coated cells were examined.

a) Formalinization after preparation

The method used was that of Fulthorpe et al. (1961). A one per cent suspension of ovalbumin coated cells was stirred continually whilst formalin was run in very slowly from a burette with its tip immersed in the cell suspension. Cells coated with bovine-serum-albumin were successfully preserved by the method in this laboratory under the writer's direction. The ovalbumin coated cells so treated were completely inactive.

b) Formalinization before preparation

(i) Formalinization. After some experimentation with the methods of Fulthorpe (1962) and of McKenna (1957), both of which were time-consuming and produced very distorted cells, the method of Csizmas (1960) was chosen. This was found to be simple, the conditions were not critical and the cells produced were undistorted and unagglutinated. Briefly, the method is to

place a dialysis sac, partly filled with formalin, in a suspension of fresh red cells. The flask is shaken for a few hours during which time saline is drawn into the sac to dilute the formalin, whilst formaldehyde gradually escapes to fix the cells. The sac is then opened to allow the contents to mix with the cells, and shaking is continued overnight. The cells are then washed well with saline (or tap water, for they are by now almost indestructible) and stored as a suspension of known concentration in saline at $4^{o}$.

Cells prepared in this way were kept for periods of a year or more without deterioration.

(ii) Tanning and coating. The formalinized cells were tanned and coated by published methods or by those described in the Methods section of this thesis. They were found to give agglutination patterns and titres corresponding to those given by fresh cells.

It was found, however, that many batches of fresh tanned cells were much more sensitive and would therefore detect smaller quantities of antibody than would the formalinized cells. Thus, with serum M0, a pool of mouse anti-ovalbumin sera, fresh cells of batch 219 gave a titre of 81,920, batch 350 gave a titre of 20,480,

batch 428 gave a titre of 20,480, and batch 490 gave a titre of 40,960, whilst formalinized cells of batch B gave a titre of 1280, batch C gave a titre of 1280, batch D gave a titre of 640, and batch K gave a titre of 5120.

(iii) The long term preservation of the formalinized cells by freezing. It was found that tanned and coated formalinized cells could be kept for days or weeks as a 1 per cent suspension at $4^{o}$ in a fully active condition without preservative. However, some batches lost titre during this time and others developed obvious bacterial or fungal contamination.

McKenna (1957) reported that formalinized cells could be frozen without damage, so this was tried. Bottles of cells from a batch coated with ovalbumin were frozen with solid $CO_2$ and acetone at $-70^{o}$ or by placing them in the deep freeze for some hours at $-20^{o}$. On testing with an anti-ovalbumin serum, cells frozen by either method or left unfrozen gave exactly the same end-point.

Later, when large batches of formalinized cells were prepared, it was found best to quick freeze the cells in glass, screw cap, Universal containers with acetone and solid $CO_2$. If they were frozen slowly at $-20^{o}$, the sedimentation

which occurred before freezing was complete caused them to stick together in lumps. These could be partly broken up by vigorous shaking, but a better suspension was produced by ultrasonicating the vial for 30 seconds in a bath used for cleaning electron microscope parts (Ultrasonic Industries Inc., Albertson, N.Y.). This did not damage the cells and tests showed that it had no effect on their sensitivity.

Excess suspension left after use was put in the deep freeze and froze slowly. It therefore had to be treated in this way with ultrasound if it was used later. As far as possible, only quick frozen suspensions were used for titrations.

After having been frozen at $-70^{\circ}$, the bottles of cells were stored at $-20^{\circ}$. There was no indication that the reactivity of ovalbumin coated cells changed during storage for periods of up to 18 months.

4. Investigation of the conditions necessary for tanning and coating formalinized sheep red blood cells

The ability to preserve tanned and coated cells over long periods, as a batch from which samples could be drawn as required, was a useful step forward.

The process of preparing a large volume of cells was laborious. There were also occasions when the cells produced only gave very low titres with standard anti-sera or auto-agglutinated or were completely inactive. It was also unfortunate that the preserved cells were less sensitive than fresh ones.

A series of experiments were therefore carried out with the object of learning something of the reasons for these variations. It was also hoped that it would be possible to devise a simple method of coating a large number of cells at one time.

The results which follow were all obtained by tanning and coating formalinized cells by a standard method, given in detail below. This was varied as made necessary by the requirements of each test.

(i) A volume of formalinized cell suspension equivalent to 0.3 ml of packed cells was placed in a 1 oz screw capped glass vial (McCartney bottle). More saline was added to half fill the bottle. It was then centrifuged at 750 G for 5 minutes at room temperature to bring down the cells. The supernate was discarded.

(ii) The cells in the vial were resuspended

in 5 ml of phosphate buffered saline (P.B.S.) pH 7.2, made up to the formula given in the Methods section. Five ml of a 1:10,000 solution of tannic acid (Mallinkrodt Chemical Corp.) in P.B.S. was added. The vial was shaken up and incubated in the 37° water bath for 15 minutes. At the end of this time, the cells were again brought down by centrifugation and the supernate removed. They were then washed once with P.B.S. and recentrifuged.

(iii) The supernate was discarded and the cells resuspended in 5 ml of P.B.S. To this was added the antigen solution, 10 mg of ovalbumin in 5 ml of P.B.S. The vial was gently shaken and placed in a 37° water bath for 30 minutes. During this time the vial was shaken once.

(iv) The cells were again spun down at 750 G and the supernate removed. They were then washed three times with P.B.S. After the last wash they were resuspended to 25 ml in P.B.S. to which had been added one part in a hundred of normal rabbit serum (N.R.S.). This serum had been well absorbed with cells of the batch under test.

(v) Batches of cells thus prepared were compared for sensitivity by titration with a rabbit anti-ovalbumin serum. To make the

comparisons accurate, the serum dilutions were first prepared in 1 ml volumes and then 0.1 ml volumes of each dilution were distributed to the wells in a plastic plate. One row of wells was filled for each batch of cells to be tested and a saline control well added. Cell suspensions from each batch were added in 0.1 ml volumes to the serum dilution rows. The plate was shaken and allowed to stand overnight at room temperature without disturbance before the end-points were read as described in the Methods section of this thesis. The batches were usually set out in a random manner on the plate to reduce any subjective influence on reading the end-points. In some cases an end-point interpolated between two dilutions has been recorded.

In the tests which follow it has usually been possible to mark one vial of cells in each as representing cells prepared under standard conditions, i.e. pH 7.2, 1:10,000 tannic acid, coating with 10 mg ovalbumin, and otherwise as described above. This vial is marked 'S'.

a) Changes in the conditions under which tanning and coating was carried out

Test 1. The effect of increasing the number of cells treated with a standard quantity of tannic acid and antigen.

Different packed cell volumes of formalinized

cells were treated by the technique described in the Introduction. This was carried out in vials of the same size but each batch of cells was finally diluted to a different volume to make a 1 per cent suspension in each case.

Table 16 shows that there was a very marked variation in the sensitivity of the cells produced when they were tested with an anti-ovalbumin serum. The more cells that were treated, the less the titre.

Table 16. Titre of anti-ovalbumin serum given by different volumes of packed cells, tanned and coated with the same quantities of materials and under the same conditions.

| Packed cell volume | Final volume of 1 per cent cells | Titre |
|---|---|---|
| 0.17 ml | 12 ml | 1,000,000 |
| S 0.35 ml | 25 ml | 102,400 |
| 0.70 ml | 50 ml | 25,600 |
| 1.4 ml | 100 ml | 200 |
| 2.8 ml | 200 ml | < 100 |

S = standard conditions.

Test 2. The effect of changes in the time for which cells were exposed to tannic acid.

Cells in five vials each containing 0.3 ml of packed cells were tanned under the standard

conditions except that the tanning time was 5, 15, 30, 60, or 120 minutes instead of the standard 15 minutes. They were then washed once and each made up to 25 ml of suspension with P.B.S. It was found that the same dilution of normal rabbit serum was required in each case to stabilize the cells and bring them down to a perfect negative button in the wells of a plastic agglutination plate. In the absence of normal serum, tanned cells, whether coated or not, auto-agglutinate and give agglutination settling patterns.

This showed that variation in the time of exposure of the cells to the tannic acid had no effect on their agglutinability.

Test 3. Investigation of the relationship between the strength of the tannic acid solution used to treat the cells and the amount of normal rabbit serum subsequently needed to stabilize them.

Formalinized cells in three vials were tanned with either 1:20,000, 1:5,000 or 1:2,500 tannic acid. After washing, each was made up to a 1 per cent suspension in P.B.S. and tested with doubling dilutions of absorbed normal rabbit serum to find out how much was required to bring them down to a perfect negative button in the wells of a plastic plate. This was found to be

1 in 80, 1 in 40 and 1 in 10 respectively. There is therefore a direct relationship between the amount of tannic acid used and the amount of serum needed for stabilization. The cells agglutinate more strongly as the concentration of tannic acid is increased.

Test 4. Investigation of the effect of varying the amount of tannic acid used to treat the cells whilst subsequently exposing them to the same amount of antigen.

Five vials of formalinized cells were treated with tannic acid concentrations from 1:80,000 to 1:5000 as shown in table 17. After washing, the cells in each vial were coated with 10 mg of ovalbumin and finally resuspended to 25 ml in P.B.S. Tests were then made on the cells to find out the most suitable concentration of normal rabbit serum to stabilize each batch.

The appropriate amount of N.R.S. was then added and the cells in each vial tested with an anti-ovalbumin serum. The results, table 17, show that with appropriate stabilization, an increased amount of tannic acid gives somewhat greater sensitivity.

Table 17. The effect of varying the amount of tannic acid used, on the titre given by coated formalinized cells.

| | Concentration of tannic acid used | Dilution of normal rabbit serum used for stabilization | Titre |
|---|---|---|---|
| | 1:80,000 | 1:150 | 10,240 |
| | 1:40,000 | 1:100 | 20,480 |
| | 1:20,000 | 1: 50 | 20,480 |
| S | 1:10,000 | 1: 50 | 40,960 |
| | 1: 5,000 | 1: 50 | 40,960 |

S = standard conditions.

Test 5. Could a concentrated suspension of tanned cells be effectively coated with antigen?

Formalinized cells were tanned in bulk by adding a large volume of them, as a 6 per cent suspension, to an equal volume of a 1:10,000 solution of tannic acid. After incubation at 37° for 15 minutes the cells were washed once. This is equivalent to the standard treatment given to the smaller batches.

After resuspension, these tanned cells were distributed in different quantities to each of four vials, so that these contained packed cell volumes of 0.17, 0.35, 0.70, and 1.40 ml respectively. The cells in each vial were spun

down, resuspended to 5 ml and coated with 10 mg of ovalbumin in 5 ml P.B.S. as in the standard method. After washing, the contents of each vial were made up to a 1 per cent suspension as in Test 1 above.

The suspensions were tested with anti-ovalbumin serum and found to give the titres shown in table 18. From these results it appears that many cells can be effectively coated in a small volume without increasing the amount of antigen available nor changing the other conditions as long as the cells have all been effectively treated with tannic acid.

Table 18. Titres of anti-ovalbumin serum given by different packed cell volumes of cells (all equally tanned) coated under the same conditions in small bottles.

| | Packed cell volume of tanned cells | Final volume of one per cent suspension | Titre |
|---|---|---|---|
| | 0.17 ml | 12 ml | 10,240 |
| S | 0.35 ml | 25 ml | 30,000 |
| | 0.70 ml | 50 ml | 20,480 |
| | 1.40 ml | 100 ml | 30,000 |

S = standard conditions.

Test 6. The effect of varying the quantity of antigen used for coating the cells.

Cells in six vials were uniformly tanned with a 1:10,000 solution of tannic acid. They were then coated with amounts of ovalbumin varying from 2.5 to 80 mg per vial before being washed and resuspended to 25 ml each in the usual way.

As shown in table 19 there was no appreciable variation between the batches when they were tested with an anti-ovalbumin serum.

Table 19. Titre of an anti-ovalbumin serum given by tanned cells exposed to different quantities of ovalbumin during coating.

| | Amount of ovalbumin used whilst coating the cells | Titre |
|---|---|---|
| | 2.5 mg | 20,480 |
| | 5.0 mg | 40,960 |
| S | 10.0 mg | 40,960 |
| | 20.0 mg | 40,960 |
| | 40.0 mg | 40,960 |
| | 80.0 mg | 20,480 |

S = standard conditions.

Test 7. The effect of varying the time during which coating was carried out.

Seven vials of cells were tanned and coated

under the standard conditions except that the time during which each was left in the 37° water-bath for coating was varied from one to 64 minutes. In the case of the vials of cells coated for only 1 and 2 minutes both the antigen solution and the cell suspension were warmed to 37° before being mixed. They were spun down at a high G force immediately the set time had expired.

The results, table 20, show that though a longer period of coating does increase the sensitivity of the cells, they were well coated by exposure to the antigen for only one minute.

Table 20. Titres of anti-ovalbumin given by cells exposed to the coating antigen for varying periods of time.

| Coating time in minutes | Titre |
|---|---|
| 1 | 10,240 |
| 2 | 20,480 |
| 4 | 30,000 |
| 8 | 30,000 |
| 16 | 30,000 |
| S 32 | 50,000 |
| 64 | 50,000 |

S = standard conditions.

Test 8. The effect of coating cells at different temperatures.

The normal procedure was followed using 1:10,000 tannic acid and 10 mg of ovalbumin for each vial. In one set of vials the coating was done at the normal pH of 7.2 and in the other at pH 6.2 (see also test 9). The cells were coated either a) at room temperature with continual agitation on a Matburn mixer;

or b) in a water bath at 37° with occasional shaking;

or c) in a water bath at 56° with occasional shaking.

Coating was carried out for 30 minutes in each case.

The cells were then washed and resuspended in the usual way and tested with an anti-ovalbumin serum to give the results shown in table 21. There was no appreciable difference between the batches of cells coated at the three temperatures and two pHs.

Table 21. Titres of an anti-ovalbumin serum given by cells coated at different temperatures and at two pHs.

| | pH | Temperature | Titre |
|---|---|---|---|
| | 7.2 | 20° | 102,400 |
| S | 7.2 | 37° | 204,800 |
| | 7.2 | 56° | 102,400 |
| | 6.2 | 20° | 102,400 |
| | 6.2 | 37° | 204,800 |
| | 6.2 | 56° | 204,800 |

S = standard conditions.

Test 9. Investigation of the influence of pH during coating.

Eight vials of cells were uniformly treated with 1:10,000 tannic acid. After washing they were resuspended in plain saline. Ten mg of ovalbumin in 0.15 M phosphate buffer at the wanted pH was added to each. After coating for 30 minutes at 37° the cells were washed and resuspended to 25 ml as usual and tested with an anti-ovalbumin serum.

The results given in table 22 show that a slight increase in sensitivity is obtained by coating the cells at a low pH.

Table 22. Titres of an anti-ovalbumin serum given by batches of cells coated at various pHs.

| | pH used | Titre |
|---|---|---|
| | 5.65 | 102,400 |
| | 6.0 | 51,200 |
| | 6.4 | 51,200 |
| | 6.8 | 51,200 |
| S | 7.2 | 51,200 |
| | 7.6 | 51,200 |
| | 8.0 | 25,600 |
| | 8.4 | 12,800 |

S = standard conditions.

b) Changes in the materials used

Test 1. A comparison of the amount of normal rabbit serum needed to stabilize different batches of formalinized cells after tanning.

Samples of four batches of cells formalinized by Csizmas' method in April 1963, June 1963, December 1963 and February 1964, were treated with 1:20,000 tannic acid in February 1964. After a single wash with P.B.S., each sample was resuspended to 25 ml and tested to find the dilution of normal rabbit serum needed to bring it down to a good negative button. In

each case this occurred at the 1:128 dilution.

There was therefore no difference between the batches of cells in their ability to be agglutinated by tannic acid.

Test 2. The sensitivity of tanned and coated cells prepared from cells formalinized at different times and by different methods.

Cells from four batches formalinized by the method of Csizmas (1960) and one batch prepared by the method of McKenna (1957) were tanned and coated as described above. A sixth batch of formalinized cells which had kindly been supplied ready tanned by Miss Marion Wood of Burroughs Wellcome and Co. Ltd. were also coated with ovalbumin at the same strength.

After being finally washed, stabilized, and resuspended to a 1 per cent suspension, the seven different batches gave the titres shown in table 23 with an anti-ovalbumin serum. Neither individual batches of cells nor their method of formalinization appear to have any influence on the results obtained. There is, however, some indication that deterioration may occur with age.

Table 23. Titre of an anti-ovalbumin serum shown by batches of cells formalinized at different times and by different methods but tanned and coated in the same way.

| Batch of cells and method of formalinization | Date prepared | Titre |
|---|---|---|
| Csizmas' method A | October 1962 | 10,240 |
| B | April 1963 | 40,960 |
| C | June 1963 | 40,960 |
| D | June 1963 | 81,920 |
| McKenna's method | March 1962 | 40,960 |
| Burroughs Wellcome | Received October 1962 | 40,960 |

(These tests were carried out in June 1963.)

Test 3. The use of different batches of tannic acid.

Three vials of cells were tanned and coated in the standard manner. In two cases Mallinkrodt tannic acid was used. One sample was from a bottle in constant use in the laboratory and the other a fresh supply (by courtesy of Dr. G. Loewi, M.R.C. Rheumatism Unit, Taplow). The third vial was tanned with tannic acid of unknown provenance found in the laboratory. It had a cruder physical appearance and gave a more deeply coloured solution than did the Mallinkrodt materials.

The cells from all three vials were found to be stabilized by the same concentration of normal rabbit serum and they gave the same end-points with an anti-ovalbumin serum.

Test 4. The effect of coating cells with different batches of ovalbumin.

Six vials of cells were tanned and coated in the standard manner. A different batch of chromatographically prepared ovalbumin was used for each. The results of titration of an anti-ovalbumin serum with the cells are given in table 24. Most batches gave the same result, although one of them, JB3, was unsatisfactory for some unknown reason.

Table 24. Titres of an anti-ovalbumin serum recorded by formalinized tanned cells coated with different batches of chromatographically prepared ovalbumin.

| Batch of ovalbumin used to coat the cells | Titre |
|---|---|
| C 25 | 20,480 |
| J 5 + 6 (known to contain conalbumin) | 20,480 |
| JB 3 | 5,120 [1] |
| JB 4 | 20,480 |
| I 2 + 3 | 20,480 |
| L | 10,240 |

[1] = the patterns collapsed to form negative buttons on being allowed to stand overnight.

Test 5. The effect of denaturing the coating antigen.

George and Vaughan (1962) reported that they were able to prepare very sensitive batches of tanned fresh cells by denaturing crystallized ovalbumin with urea before coating the cells.

This method of denaturation and two others were therefore tested.

a) Urea. A solution of ovalbumin containing 20 mg in 10 ml of saline was prepared. Solid urea was then added to a final concentration of 6 M. After 1 hour at 37° and 7 hours at room temperature the urea was dialysed away overnight with running tap water.

b) Surface denaturation. The ovalbumin solution was put in a container of a size such that ample free space was available (e.g. 10 ml in a 25 ml bottle). It was then placed on a Matburn blood cell suspension mixer for 3½ hours at room temperature. Due to the shaking which occurred, long strings of denatured ovalbumin appeared in the solution during this time.

c) Heat. The ovalbumin solution was placed in a 56° water bath for 3½ hours. During this time a fine cloudiness appeared.

Three vials of cells were tanned and coated by the standard method using these preparations

of denatured ovalbumin. A fourth vial of cells was coated with normal ovalbumin.

On testing with an anti-ovalbumin serum titres were obtained which are given in table 25. These do not confirm the results of George and Vaughan so far as chromatographically prepared ovalbumin and preserved cells are concerned. The cells coated with the urea denatured material gave the lowest titres. These relatively small variations may not, however, have any great significance.

Table 25. Titres of an anti-ovalbumin serum given by formalinized cells coated with ovalbumin which had been denatured by various means.

| Method of denaturation | Titre |
|---|---|
| 6 M urea | 2,560 |
| Shaking at room temperature | 10,240 |
| Heating at 56° | 40,960 |
| Normal, undenatured ovalbumin | 10,240 |

c) Conclusions

These tests showed that the efficiency with which the cells were tanned was the chief factor which affected the sensitivity of the finished product to antibody. The quantity of antigen,

the pH, time, temperature, and the batches of materials used were of relatively minor importance.

In designing a simple method for the preparation of a large volume of cells it was therefore possible to ignore many factors so long as the tanning process was efficient. It was found possible to carry out all the processes on a very thick suspension (about 25 per cent) of packed cells in a McCartney bottle. This was easy to handle in a bench centrifuge and large volumes of buffer and so forth were not required.

The cells were tanned with an adequately large amount of tannic acid. It was found to be important to ensure that this was mixed in very rapidly to ensure that all the cells were evenly treated, otherwise some cells were overtanned and others unaffected.

It was not found necessary to use large amounts of antigen to coat the cells nor to increase the times of treatment excessively from those outlined in the method given above. Adequate mixing and resuspension at all stages was important.

After washing had been completed in the small vial, the cells were resuspended to make 500 ml of a 1 per cent suspension in P.B.S. Up

to this point no normal rabbit serum was added; another considerable economy. The suspension was then tested with dilutions of normal rabbit serum to find that which was most suitable for stabilization. After addition of the correct amount to the bulk suspension, the cells were bottled in small quantities and quick frozen with acetone and solid $CO_2$ before being stored at $-20^{o}$. Full details of the process are given in the Methods section of this thesis.

## C. DISCUSSION

### 1. The influence of conalbumin on the tanned red cell agglutination test

An early paper reporting experiments with Boyden's tanned red cell agglutination test (1951) was that of Borduas and Grabar (1953). Amongst other findings they reported that when ovalbumin-coated red cells were used to titrate anti-ovalbumin antibodies they actually recorded antibodies to conalbumin, a minor impurity in the ovalbumin. If this report was correct, it would have a most damaging effect on any pretension to specificity that the tanned cell test might have with this or other antigen-antibody systems.

Borduas and Grabar based their report on the following results. Two batches of cells were prepared, one sensitized with ovalbumin (shown to contain some conalbumin by immunological examination) and the other sensitized with conalbumin (not characterized). They then measured the dilution of a 0.1 per cent solution of either antigen which it was necessary to add to a rabbit anti-ovalbumin serum to inhibit its ability to agglutinate these cells. They found that agglutination of the ovalbumin-coated cells could be inhibited by a $1:10^{-4}$ dilution of conalbumin or a $1:10^{-3}$ dilution of ovalbumin, i.e. more

ovalbumin than conalbumin was needed. The agglutination of conalbumin-coated cells was inhibited by a $1:10^{-4}$ dilution of conalbumin or a $1:10^{-3}$ dilution of ovalbumin. As before, more ovalbumin than conalbumin was needed to inhibit the reaction.

These results suggested that both batches of cells were reacting to antibodies to some substance which was about ten times greater in quantity in the conalbumin than in the ovalbumin as so much less conalbumin was required to absorb it from the serum. Borduas and Grabar interpreted this to mean that the cells were reacting to anti-conalbumin rather than to anti-ovalbumin and that the cells were preferentially coated with conalbumin. Some additional evidence arose from the fact that the cells had been found to be optimally coated (to react with this serum) by much less conalbumin than ovalbumin.

This rather alarming report by Borduas and Grabar caused two other investigators, who were working with ovalbumin and tanned red cells, to make some checks of its validity.

Stavitsky (1954b) examined conalbumin and ovalbumin preparations which had been recrystallized 6 and 8 times respectively. He found that each was still contaminated with the

other. These preparations were used in tanned cell agglutination inhibition tests with an anti-ovalbumin serum which contained antibodies to both of them (sic). The results indicated that the ovalbumin-anti-ovalbumin system was less sensitive than the conalbumin-anti-conalbumin system but false results did not seem to be a danger.

George and Vaughan (1962) also decided that conalbumin was not a dangerous contaminant of ovalbumin. An anti-conalbumin serum prepared by them and known to contain anti-ovalbumin antibodies was completely inactivated toward ovalbumin-coated tanned cells after absorption with optimal amounts of ovalbumin. This they interpreted as meaning that conalbumin and anti-conalbumin had no role in the reaction.

The present experiments, the results of which are analysed in detail on a previous page, show that cells coated with a mixture of chromatographically prepared ovalbumin and conalbumin react to the major antibody present in the serum. They are not more sensitive to anti-conalbumin than to anti-ovalbumin. It was also apparent that the cells could be effectively sensitized by a mere trace of ovalbumin present as an impurity in conalbumin. It may well be

that the converse also occurs but no tests were made.

There was no evidence that any masking of ovalbumin by conalbumin occurs such as that reported by Linz and Lecocq (1962). They found that a 'weak' antigen (bovine serum albumin) might be well adsorbed by the cells but masked by a 'strong' antigen (such as human or horse serum) if this was present at the same time. The cells could then be only agglutinated by sera against the 'strong' antigen.

Another finding in the present work was that rabbit sera raised against twice recrystallized ovalbumin contained a high level of antibody to conalbumin. This may help to explain the findings of Borduas and Grabar.

The serum tested by Borduas and Grabar had been prepared with a crystalline ovalbumin preparation known to contain conalbumin. This serum was not characterized by immunological methods and it may have contained a high level of anti-conalbumin. If so, this would adequately account for the results obtained. Their inhibition test was carried out with tenfold antigen dilution steps and only single dilution differences were found. It would not therefore be necessary to have very much more anti-conal-

bumin than anti-ovalbumin in the serum to reverse the interpretation of the reported readings if it is assumed that each batch of cells was responding to both antibodies.

The lesson of this work appears to be that well characterized antigens and antibodies should be used. Often a simple overnight gel diffusion test will be sufficient to give adequate information on this point.

2. The optimum conditions for tanning and coating formalinized sheep red blood cells

Boyden's original development of a passive agglutination test using tanned red cells (1951) arose from investigations which he was making into the agglutination of red cells by tannic acid. The rationale of the test is that the tanning process makes a normally non-agglutinating reaction into an agglutination one by causing surface changes on the red cells (Pirofsky et al., 1962), not that it causes antigens to stick on the cells.

Sindo and Wakakura (1952) found that sheep red cells would adsorb ovalbumin and so become susceptible to agglutination or lysis by antisera even if they were not tanned. In an experiment not detailed above, the author confirmed that

formalinized sheep cells could be coated with ovalbumin even though they were untanned. These cells only showed very low titres with strong antisera.

The effect of tanning is therefore to increase the agglutinability of the red cells and so make the reaction more sensitive. Adequate tanning is thus a pre-requisite for the preparation of active batches of cells (compare table 16 with table 18). The results reported show that the more tannic acid that was used the more agglutinable the cells became (test a.3) and the more sensitive was the final product (table 17).

However, the use of more tannic acid was not all gain, for more normal rabbit serum was then required to stabilize the cells (table 17) and this would presumably reduce their actual sensitivity. This is a very important factor in the tanned cell test. An essential part of the preparation of cells for the test is their stabilization with a small quantity of normal rabbit serum so that they will settle to a non-agglutination pattern when no antibody is present. On the other hand, in the test proper more serum, with stabilizing action, is added in the low dilutions of the serum being tested. Therefore in the low dilutions the stabilizing action may

be greater than the agglutinative action of the antibody present and artificially low titres will be recorded. The reality of this effect could be tested for by adding low dilutions of normal serum to the tubes in the end-point area of a test of a high titred serum.

The key to the preparation of tanned cells as a concentrated suspension rather than the dilute 1-2 per cent suspension usually used, lay in the adequate tanning of each cell rather than the alteration of any other item of treatment.

Finally, no normal rabbit serum was used to wash the cells after coating. After they had been made up to the normal 1 per cent suspension for use they were titrated with normal rabbit serum. This titration showed the minimum quantity which would be just sufficient to bring the cells down to a negative button if no antibody was present and this was added to the batch. Maximum sensitivity was thus preserved.

Tests were also carried out in which the conditions used for tanning and coating the cells were varied. Most of the published work in this field had been concerned with fresh cells and formalinized cells may not react in a similar manner. Comparisons between them may not, therefore, be valid.

Thus, in the present tests, formalinized cells were found to be almost as well coated by a solution containing 0.5 mg/ml of ovalbumin as they were by a solution containing 16 mg/ml (table 19). However, using fresh cells, George and Vaughan (1962) found that there was a direct relationship between the quantity of antigen which was used to coat the cells (0.1 - 1.0 mg/ml) and their eventual sensitivity. The author confirmed that this was true for fresh cells. Antigen solutions containing 0.5, 1.0 or 2.0 mg/ml of crystallized ovalbumin were used to coat three batches of fresh cells. These cells subsequently recorded titres of 20,480, 40,960, and 163,840 respectively, for the same serum.

Even with fresh cells different authors have reported wide variations between the optimum concentration of ovalbumin needed to sensitize the cells. Boyden (1951) found that 5 mg/ml was needed for maximum sensitivity whilst Steffen and Rosak (1963) found maximum sensitivity with 0.3 mg/ml. More or less than this was much less effective. However, Linz et al. (1961) in their work with several other antigens found that the concentration of antigen needed to sensitize the cells could be varied widely without altering the eventual sensitivity of the resultant cells. This they considered to be due to the fact that

only a very small part of the total protein present was actually adsorbed on to the cells, though this did seem to be related to the total amount present.

George and Vaughan (1962) suggested that the variations between the sensitivity of different batches of cells coated with ovalbumin might be explained by assuming that antigen was denatured to a different extent in each case. They were able to show that coating fresh cells with urea-denatured crystallized ovalbumin gave them greater sensitivity. Denaturation of the antigen was tried in the present work but only material heated to 56° for 3½ hours seemed to sensitize the cells more fully (table 25). This result may be related to the finding of Christian (1958) that heat aggregated gamma globulin was a better coating agent for tanned cells than was non-aggregated material. George and Vaughan also found that the active coating material from urea denatured ovalbumin was found in the pellet after ultracentrifugation.

Urea denaturation was not found to be effective (table 25). This failure to confirm the results of George and Vaughan cannot be due to some physical difference between chromatographically prepared ovalbumin and that prepared

by the normal method and it is unlikely that any impurity was present in the material used by George and Vaughan. A sample of ovalbumin, not fully characterized, but prepared by them using two types of chromatography followed by electrophoresis also had its sensitizing ability for red cells increased by denaturation.

In the present work, the time of exposure to tannic acid, the quantity of antigen used, and the temperature at the time of coating had no effect on the resultant sensitivity of the cells. However, by coating the formalinized cells at a low pH (table 22) or for a long period of time (table 20) some increase in sensitivity was obtained though the overall differences were not very great.

Both Stavitsky (1954a) and Boyden (1951) coated protein antigens on to the cells at pH 6.4 which Boyden had found to be an optimum. Tanning was carried out at pH 7.2. Most authors have followed this procedure without reporting any tests to find an optimum. However, Felton et al. (1961) working with herpes simplex virus, a very different antigen than the purified proteins usually employed in immunological work, found that they were unable to sensitize fresh cells at a pH above 7.0 and that the optimum was

pH 6.4. There was no sensitization if the cells were exposed to the antigen for less than 10 minutes though a longer exposure was not advantageous.

In the present work, the use of different batches of tannic acid, or of ovalbumin was found to have no effect on the sensitivity of the cells (test b.3, and table 24). There did seem to be some deterioration of the older batches of formalinized cells which had been stored without preservative at $4^{o}$ before treatment (table 23).

The cells were formalinized by the method of Csizmas (1960). This method was very easy to carry out with stock reagents from the laboratory shelves. It was not necessary to neutralize the formalin (Weinbach, 1958) and formalinization was completed overnight rather than over a period of ten days (Ingraham, 1958). The cells produced were ready for immediate use and did not have to be dispersed from clumps with a homogeniser (Cole and Farrell, 1955) nor treated with sodium bisulphite (McKenna, 1957). The cells produced were normally shaped, un-crenellated, almost indestructible and it was not necessary to add any protective glycerol when freezing them (Fahey and Humphrey, 1962).

The finished suspension was stored in saline

at $4^{o}$ without the addition of any Merthiolate (Weinbach, 1958) or formalin (Ingraham, 1958). The preserved cells seemed to be very formalin-free and slow damage by contaminating organisms may have occurred as no sterile precautions were taken with them. If a stock of cells is to be kept for a long period it may therefore be wise to add some preservative to prevent any deterioration occurring.

Although a method was evolved for the easy preparation and storage of formalinized tanned cells these appeared to be intrinsically less sensitive than fresh ones. However, their great convenience as a standard reagent and the simplicity and reliability of the test carried out with them, commends them as a first-class method of antibody titration in any system to which they can be applied.

# Part Five

## Experimental

## THE MINERAL OIL ADJUVANTS

## Preliminary Notes

The results of the experiments carried out with adjuvants have been arranged in five groups as follows:



A chief aim in all of these experiments was to find reasons for the action of this type of adjuvant. There is therefore some overlapping between the groups.

Titrations were usually carried out with tanned and coated formalinized red cells, stored frozen. When very low titres were expected, fresh cells were used as these were found to be up to 64-fold more sensitive.

Different batches of tanned cells vary in

their sensitivity. By checking one batch against another their inter-relationships were established. All the titres quoted have been adjusted to those which would have been given by a standard batch of formalinized cells. Thus some low titres read with fresh cells have had to be divided by factors up to 64. Hence the mean titres of less than unity.

## A. Characterization of the normal response of mice to an antigen given in a water-in-oil emulsion



### 1. The choice of a suitable test dose of ovalbumin to be used in mice

To avoid immunological paralysis due to the use of too large a dose of antigen or the lack of a response due to too little, it was necessary to choose the lowest dose of ovalbumin which would raise an easily measurable level of antibodies.

The route of administration was subcutaneous and for these preliminary experiments saline was used as the vehicle for the antigen.

#### a) Using twice recrystallized ovalbumin (commercial source)

Mice in four groups of ten were each given 0.5, 1.0, 2.0, or 4.0 mg of ovalbumin dissolved

in saline and sterilised by Seitz filtration. Another eight mice were left uninoculated.

Serum samples were collected on the days shown in table 26. These were titrated with fresh red cells which had been coated with the same crystalline ovalbumin used for the inoculations.

On the 39th day after the initial inoculation the mice in the groups given 0.5, and 2.0 mg were each given a secondary dose of 1.0 mg of the same ovalbumin in saline. ' The uninoculated mice were also given 1.0 mg of ovalbumin at this time. Three further samples were collected from the mice in these groups.

The geometric mean titres for each group are shown in table 26 and figure 39 shows these results graphically. A dose response curve is plotted in figure 41 for days 19 and 22 of the primary response.

Of the mice in the groups given 1.0 and 4.0 mg ovalbumin, only three of ten animals showed detectable antibodies. In the 2 mg group, seven out of ten reacted to the primary stimulus and all reacted to the secondary.

Two milligrams of ovalbumin was therefore chosen as the standard test dose.

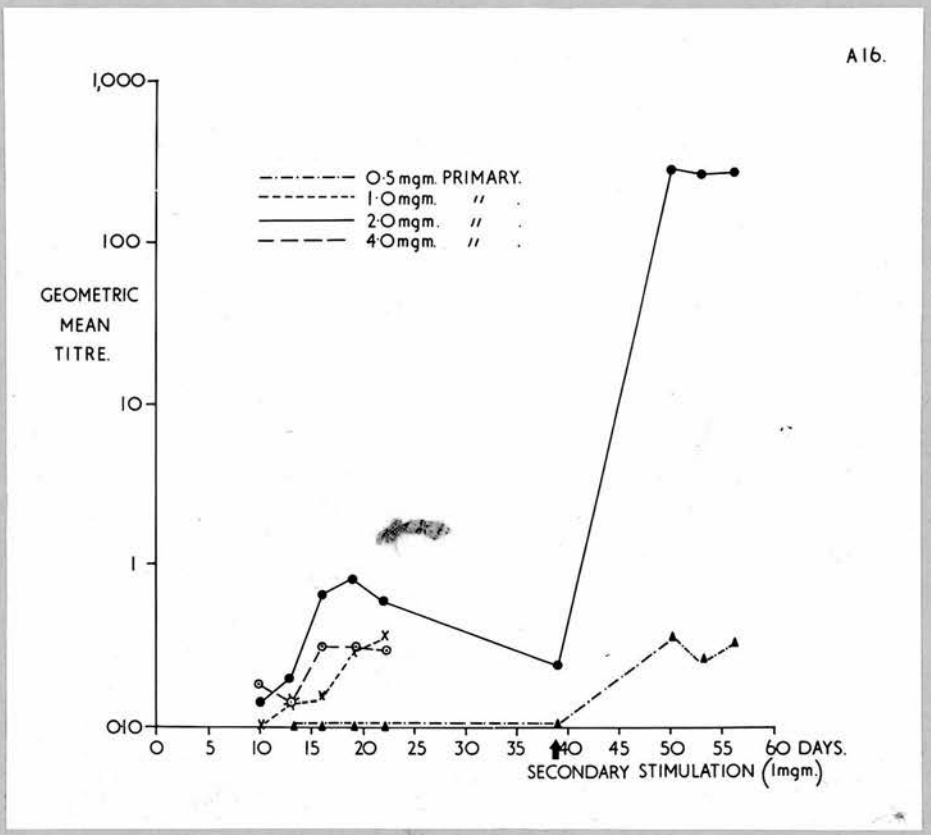



Fig.39. The antibody responses of groups of mice inoculated with primary doses of 0.5, 1.0, 2.0 or 4.0mg of crystalline ovalbumin in saline. A secondary stimulation of 1.0 mg of crystalline ovalbumin in saline was given on the 39th day.

Table 26. Geometric mean antibody titres of groups of mice given various doses of ovalbumin in saline.

| Dose | Number of mice (Grey) | Mean weight | Days after first injection | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 10 | 13 | 16 | 19 | 22 | 39 | 39 | 50 | 53 | 56 |
| Nil | 8 | 27.2 g | 0 | ND | 0 | 0 | 0 | 0 | 0 | S | 0 | 0.28 | 0 |
| 0.5 mg | 10 | 25.3 g | 0 | ND | 0 | 0 | 0 | 0 | 0 | S | 0.35 | 0.26 | 0.32 |
| 1.0 mg | 10 | 25.5 g | 0 | 0 | 0.14 | 0.15 | 0.30 | 0.35 | - | - | - | - | - |
| 2.0 mg | 10 | 26.5 g | 0 | 0.14 | 0.20 | 0.65 | 0.82 | 0.60 | 0.24 | S | 280 | 259 | 261 |
| 4.0 mg | 10 | 29.0 g | 0 | 0.18 | 0.14 | 0.31 | 0.31 | 0.29 | - | - | - | - | - |

ND = not done. S = secondary injection of 1.0 mg of ovalbumin in saline.

- = no sera collected. 0 = no antibodies detected.

b) Chromatographically prepared ovalbumin

Later, it was decided that chromatographically prepared ovalbumin should be used in the experiments with adjuvants. This material was tested as above at the two dose levels of 1.0 and 2.0 mg per mouse. On the 31st day secondary doses of 1.0 mg were given.

The results of titrations using cells coated with chromatographically prepared ovalbumin are given in table 27 and are shown as a graph in figure 40. The results of this experiment are also shown on the dose response curve for crystalline ovalbumin, figure 41. These graphs show that the results follow those for crystalline ovalbumin. No change was therefore made in the decision to use 2 mg as a standard test dose.

Reference should also be made to table 57. This gives the results of an experiment in which doses of ovalbumin from 0.25 µg to 1280 µg in saline were tested in mice.

Table 27. Geometric mean antibody titres of groups of mice inoculated with chromatographically prepared ovalbumin in saline.

| Dose | Number of mice (Grey) | Days after first injection | | | | | |
|---|---|---|---|---|---|---|---|
| | | 19 | 22 | 31 | 38 | 41 | 44 |
| 1 mg | 6 | 0.44 | 0.31 | S | 71.4 | 58.9 | 58.9 |
| 2 mg | 6 | 0.49 | 0.50 | S | 699 | 849 | 571 |

S = secondary injection of 1.0 mg of ovalbumin in saline.

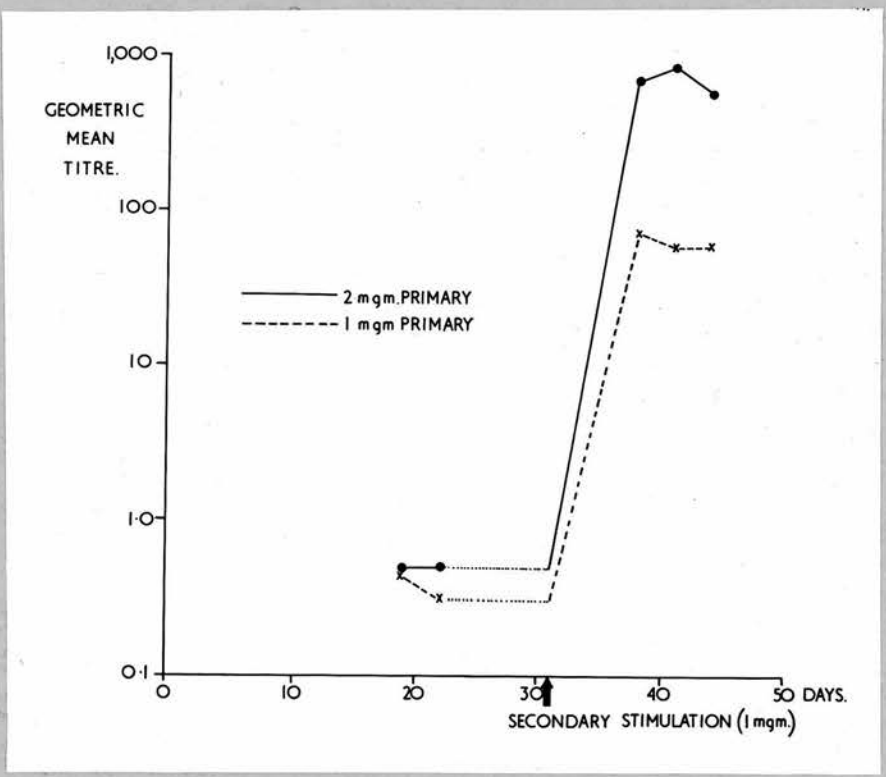



Fig.40. The antibody responses of groups of mice inoculated with 1.0 and 2.0 mg of chromatographically prepared ovalbumin in saline. A secondary stimulation with 1.0 mg of the same ovalbumin in saline was given on the 31st day.

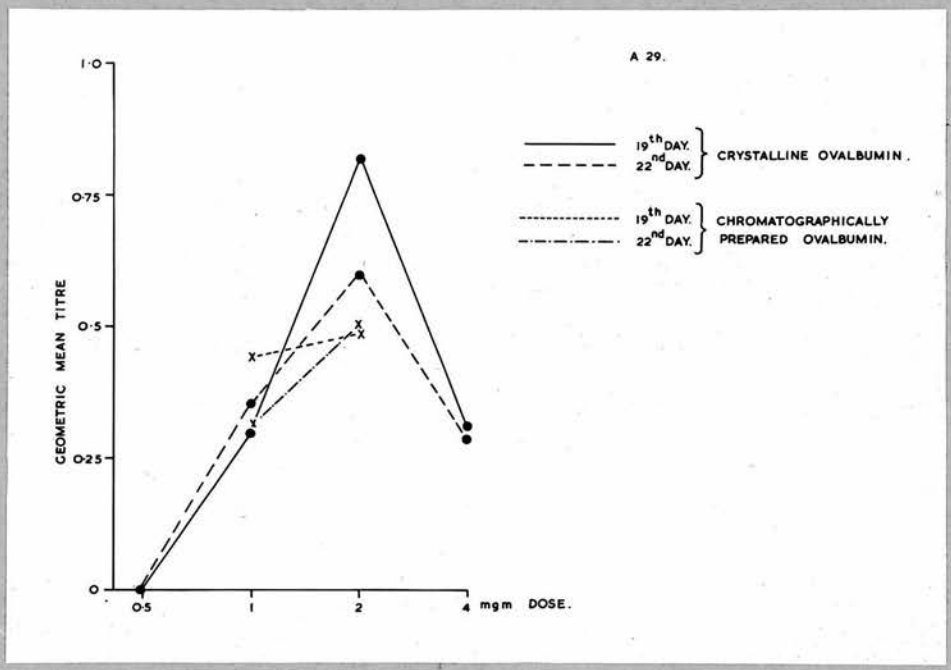



**Fig.41. Primary dose response curves for groups of mice inoculated with various doses of crystalline or chromatographically prepared ovalbumin in saline.**

## 2. The antibody response of mice given ovalbumin intravenously

A known base-line antibody response record was required for mice given antigen intravenously. Each mouse in four groups of six were therefore given 2, 4, 8, or 16 mg of ovalbumin by the intravenous route. The ovalbumin was dissolved in saline, Seitz filtered and injected into the tail veins of un-anaesthetized mice. The volume used was 0.2 ml in each case.

Four out of six mice in the 2 mg and 4 mg groups, and five out of six in the 8 mg and 16 mg groups showed a measurable response. Table 28 gives the geometric mean titres of serum samples collected from individual mice in each group. A dose response curve is plotted in figure 42.

These results showed that for the 2 mg and 4 mg doses, there was no significant difference ($P = 0.9 - 0.8$ and $0.7 - 0.6$) between the responses of mice given the antigen by the subcutaneous or intravenous routes (cf. table 26). However it must be remembered that these measurements were made at the limit of sensitivity of the titration technique and it is difficult to judge their reliability.

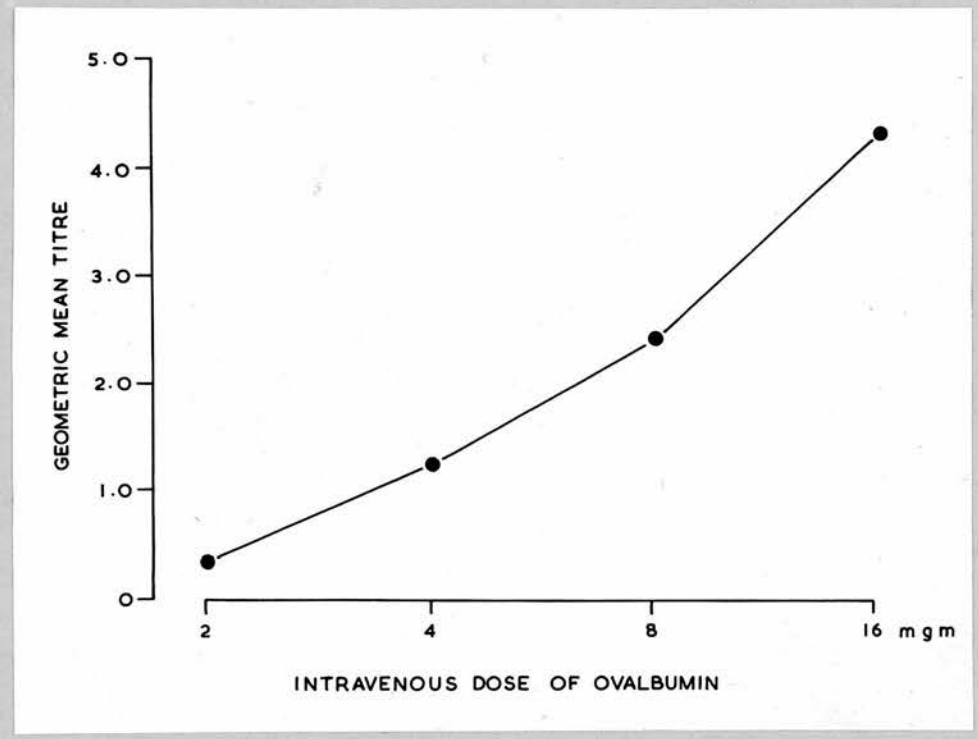



Fig.42. Dose response curve on the 25th day after injection for groups of mice given one dose of 2, 4, 8, or 16 mg of ovalbumin intravenously in saline.

Table 28. Geometric mean titres of groups of mice given ovalbumin intravenously in saline.

| Dose | Number of mice (White) | Mean weight | Days after injection | | | | |
|---|---|---|---|---|---|---|---|
| | | | 15 | 20 | 25 | 36 | 37 |
| 2 mg | 6 | 25.5 g | ND | ND | 0.32 | ND | ND |
| 4 mg | 6 | 26.9 g | ND | ND | 1.24 | ND | 0.56 |
| 8 mg | 6 | 26.6 g | 0.65 | 2.36 | 2.41 | 0.93 | ND |
| 16 mg | 6 | 28.6 g | 0.50 | 1.46 | 4.30 | 4.82 | ND |

ND = not done.

3. The antibody response of mice given ovalbumin in a water-in-oil emulsion

Six mice were inoculated subcutaneously with ovalbumin in a water-in-oil emulsion prepared with the oil Drakeol 6-VR using Aquaphor as emulsifier.

The Aquaphor was heated until melted and 0.4 ml of it placed in a sterile mortar. To this was added 16 mg of ovalbumin which had been dissolved in 0.45 ml of saline and Seitz filtered. The two were blended together with a pestle until all of the water phase had been absorbed into the Aquaphor to form a smooth cream. Drakeol 6-VR was then added in two portions of 0.4 ml each and blended into the

mixture. The resultant emulsion did not disperse when a portion was dropped into water and it was considered to be a good water-in-oil emulsion.

The mice were each given 0.2 ml of the emulsion (corresponding to 2 mg ovalbumin) subcutaneously over the back as a primary stimulation. Thirty-one days later they were each given a secondary inoculation of 1 mg of ovalbumin in saline.

Sera were collected from individual mice at intervals and titrated with tanned cells. Geometric mean titres of the results are given in table 29, and are shown graphically in figure 43. The graph shows that the titres were still rising on the 31st day when the secondary injection was given.

There was surprisingly little difference in the height of the secondary responses as shown on this graph (fig. 43) in comparison with the primary responses to ovalbumin in the various vehicles which varied widely. Student's *t* tests of the 38th day means showed, however, that the secondary response after a primary of ovalbumin in normal saline was significantly lower than the others.

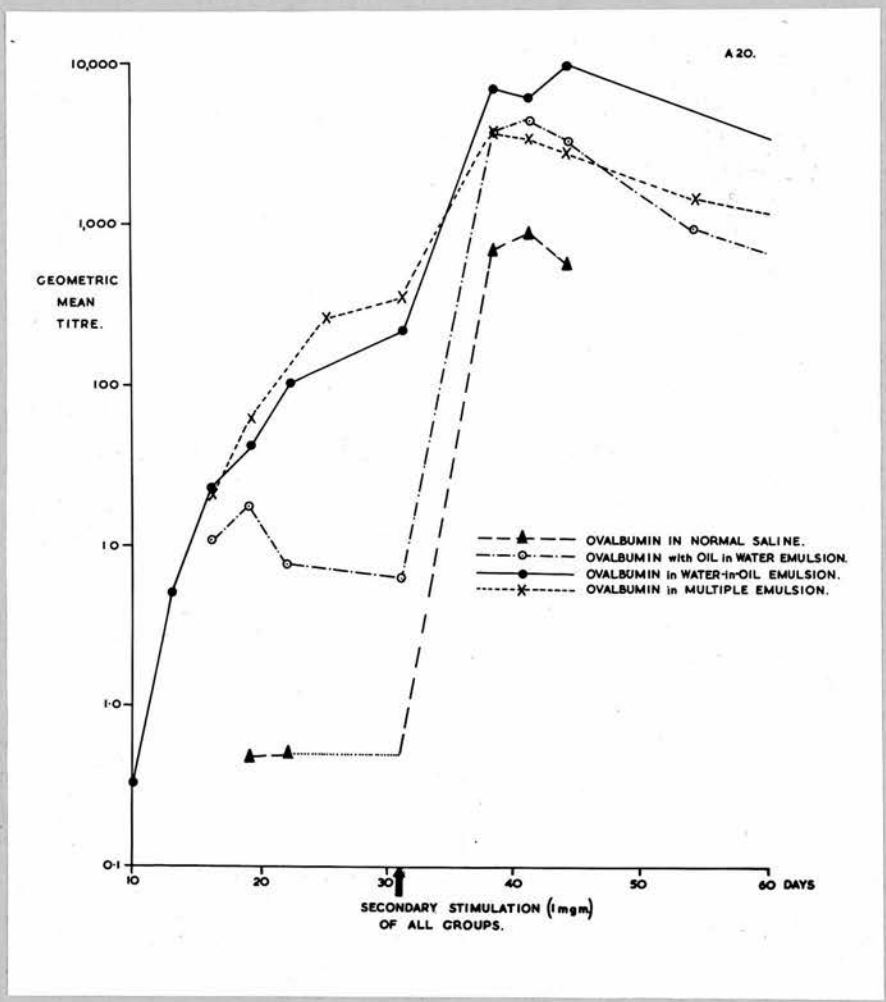



Fig.43. The antibody responses of groups of mice inoculated with 2 mg of ovalbumin in saline, water-in-oil emulsion, oil-in-water emulsion or in a multiple emulsion. A secondary stimulation with 1 mg of ovalbumin was given on the 31st day.

P = 0.01 - 0.001 for water-in-oil emulsion and saline.

P = 0.01 - 0.001 for oil-in-water emulsion and saline.

P = $< 0.001$ for multiple emulsion and saline.

The primary response curve for ovalbumin in water-in-oil emulsion shows the steady rise to a high titre typical of the response of mice given a soluble antigen in this adjuvant. The curve in its fully developed form, with a long plateau following the rise, is better seen in figures 45, 51, and 54.

4. The response of mice to various doses of ovalbumin in a water-in-oil emulsion

The object of this experiment was to find out how small a dose of ovalbumin was still effective as an antigen when given in water-in-oil emulsion.

Four water-in-oil emulsions were prepared which contained respectively 2, 20, 200 and 1333 µg of ovalbumin in each 0.2 ml of emulsion. Mice in four groups of ten were each inoculated with 0.2 ml of emulsion subcutaneously. Serum samples were collected from them on the 20th, 31st and 60th days. The results of titrations of these sera are given in table 30 and a dose

Table 29. Geometric mean titres of six grey mice each given 2 mg of ovalbumin in a water-in-oil emulsion.

| Mean weight of the mice | Days after inoculation | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 10 | 13 | 16 | 19 | 22 | 31 | 31 | 38 | 41 | 44 | 82 |
| 31.7 g | 0 | 0.32 | 5.0 | 22.4 | 40.0 | 100 | 209 | S | 6870 | 6070 | 9856 | 810 |

S = secondary injection of 1.0 mg ovalbumin in saline.

Table 30. Geometric mean titres of groups of mice given 2, 20, 200, or 1333 µg of ovalbumin each in water-in-oil emulsion.

| Dose per mouse | Number of mice (White) | Mean weight | Days after inoculation | | |
|---|---|---|---|---|---|
| | | | 20 | 31 | 60 |
| 2 µg | 10 | 32.5 g | 12.0 | 47.5 | 85 |
| 20 µg | 10 | 32.8 g | 69.5 | 278 | 508 |
| 200 µg | 10 | 32.8 g | 187 | 1522 | 1901 |
| 1333 µg | 10 | 33.6 g | 49.5 | 367 | 1280 |

response curve is plotted in figure 44. A graph of the results for the 2 µg and 20 µg groups is also shown in figure 71.

Many individuals in one group of mice, those given 200 µg, developed abscesses at the injection site. These and the original emulsion were found to be contaminated with cocci which were probably Gaffkya tetragena.

Student's t tests were carried out on the results for the 31st day. The probabilities of the different groups coming from the same population were as follows:

| | | | |
|---|---|---|---|
| 2 µg group and | 20 µg group | P = 0.05 -0.02 |
| Do. | 200 µg group | P = 0.001 |
| Do. | 1333 µg group | P = 0.005-0.0025 |
| 20 µg group and | 200 µg group | P = 0.01 |
| Do. | 1333 µg group | P = 0.6 -0.7 |
| 200 µg group and | Do. | P = 0.005-0.0025 |

The group given 200 µg each seem to be unrelated to any of the others. This may indicate some additional adjuvant effect from the Gaffkya tetragena infection or from the inflammatory processes themselves at the site of injection and infection.

The results for the other groups show that even a dose as low as 2 µgm of ovalbumin in water-in-oil emulsion will produce a reasonable

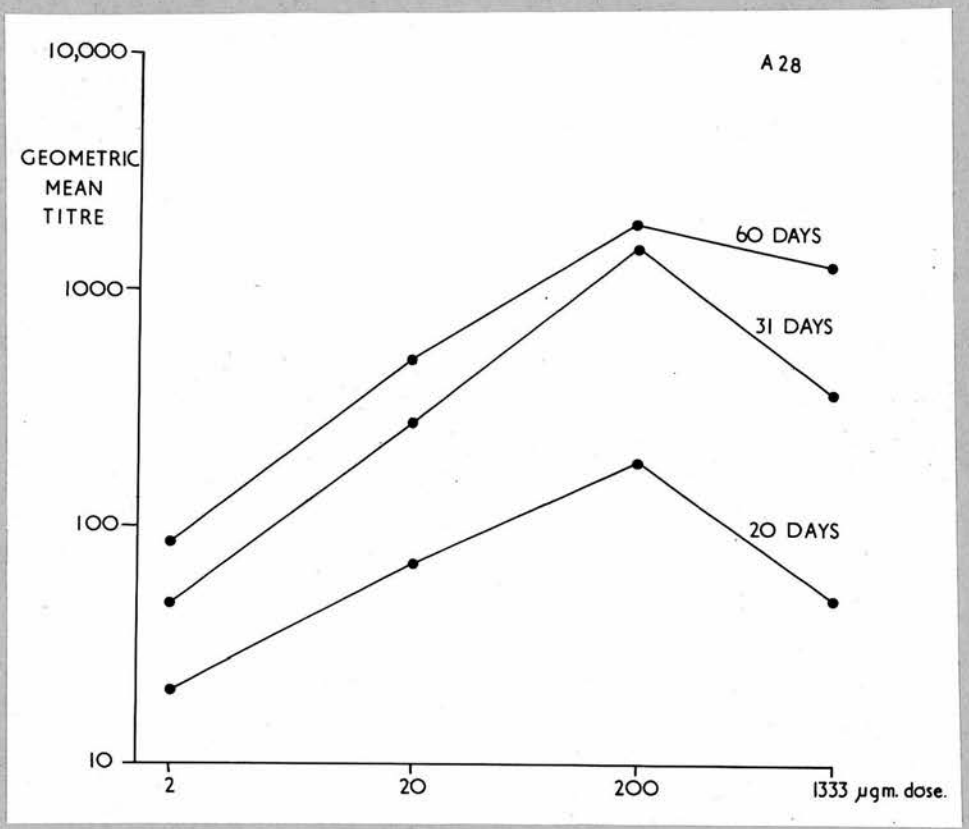



Fig.44. Dose response curves for groups of mice inoculated with ovalbumin in water-in-oil emulsion. Plotted on the 20th, 31st and 60th days after injection.

response. It is doubtful whether any antibodies would be detectable, even with the most sensitive cells, if this dose was given in saline (cf. table 57).

B. The effect of later injections of antigen on the normal response to an antigen given in water-in-oil emulsion



1. Investigation of the effect of secondary injections of ovalbumin in saline, given after an initial inoculation with ovalbumin in water-in-oil emulsion

In prophylactic immunization procedures it is common practice to give more than one injection of an antigen in order to obtain a 'booster' effect. If a depot forming adjuvant is actually releasing antigen over a long period secondary injections would appear to be superfluous. If some more subtle mechanism is stimulating antibody production a secondary injection might cause some change to occur which would be reflected in the shape of the antibody response curve.

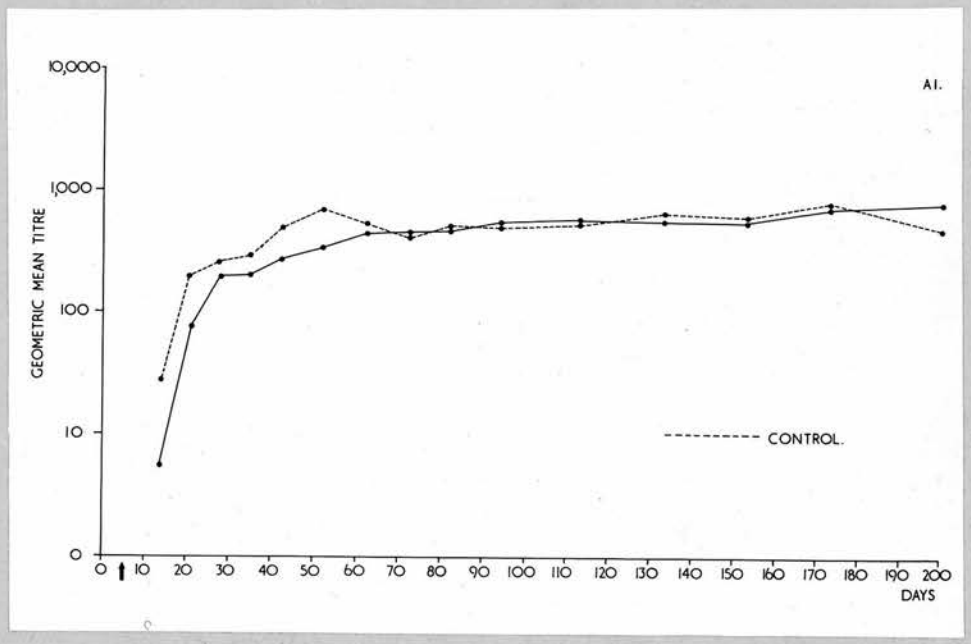
To test these ideas, groups of mice were given a single secondary injection of ovalbumin between the 5th and 129th days after a primary inoculation of ovalbumin in a water-in-oil

emulsion.

A water-in-oil emulsion containing 2 mg ovalbumin per 0.2 ml was prepared using a syringe as described in the Methods section. Seventy mice were each given 0.2 ml of the emulsion subcutaneously on the same day. The mice were then divided into seven groups one of which was to be kept as a control. The mice in the other groups were each given a single secondary injection of ovalbumin in saline on the 5th, 10th, 20th, 40th, 80th or 129th days.

Sera were collected at intervals and titrated with formalinized tanned cells. The results of these titrations are given in table 31 and are shown graphically in figures 45, 46 and 47. In each graph the curve for one of the groups given a secondary injection is shown together with the curve for the control group. There is some evidence from the curves that the secondary response occurs as a transitory incident on the basic pattern of response to antigen in water-in-oil emulsion. Secondary injections given on the fifth and tenth days had no effect on the pattern of response.

As the curves are plotted on semi-logarithmic graph paper it is possible to compare the secondary responses directly by tracing the



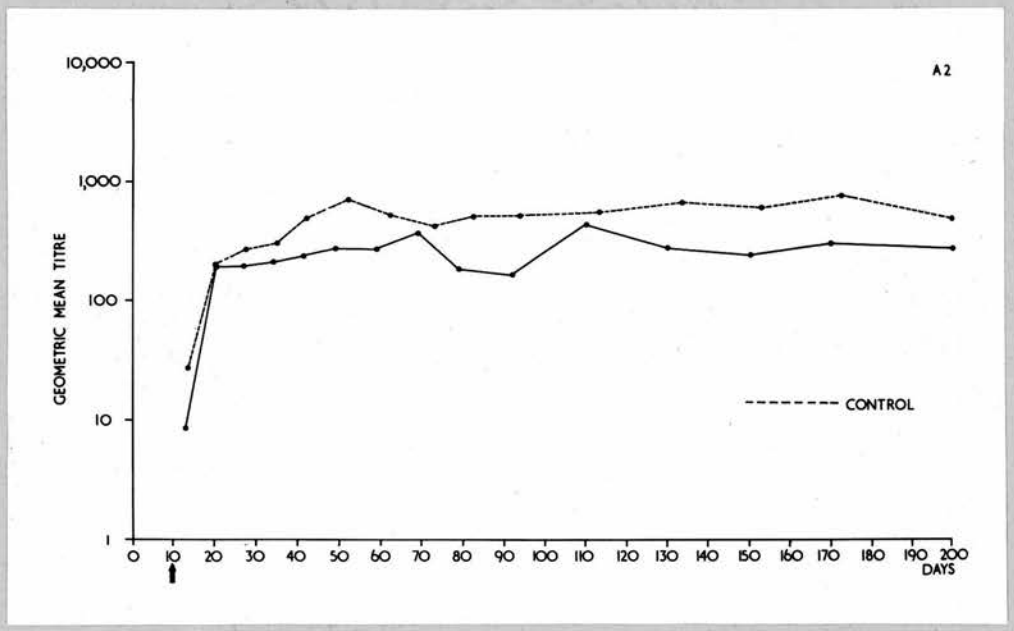
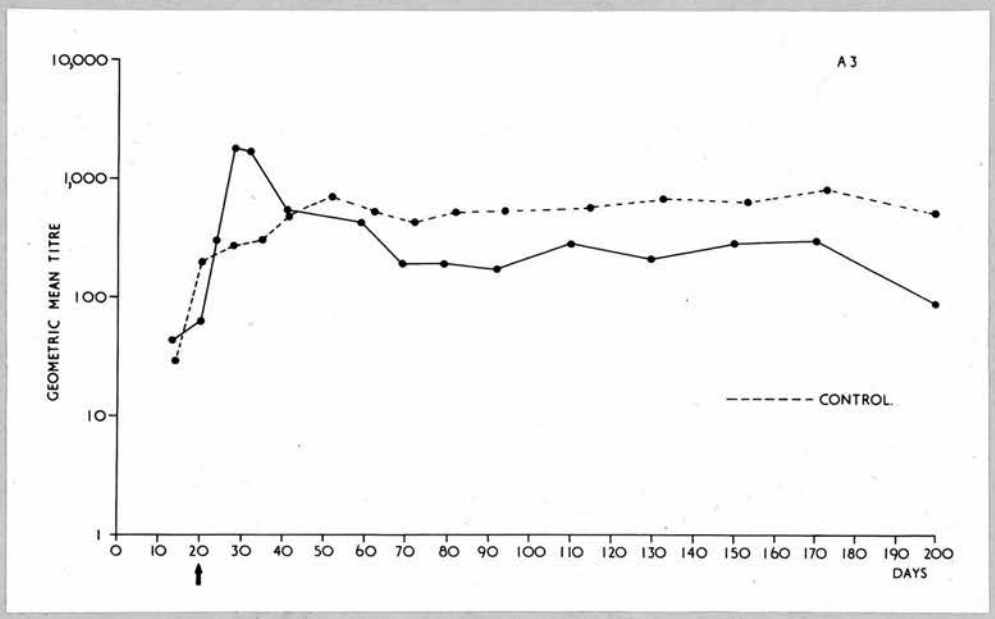




Fig.45. Antibody response curves of groups of mice given a secondary injection of 1 mg of ovalbumin in saline five days after (above) and ten days after (below) being inoculated with 2 mg of ovalbumin in a water-in-oil emulsion. The broken line on each graph is the response of a control group of mice given 2 mg of ovalbumin in water-in-oil emulsion only.



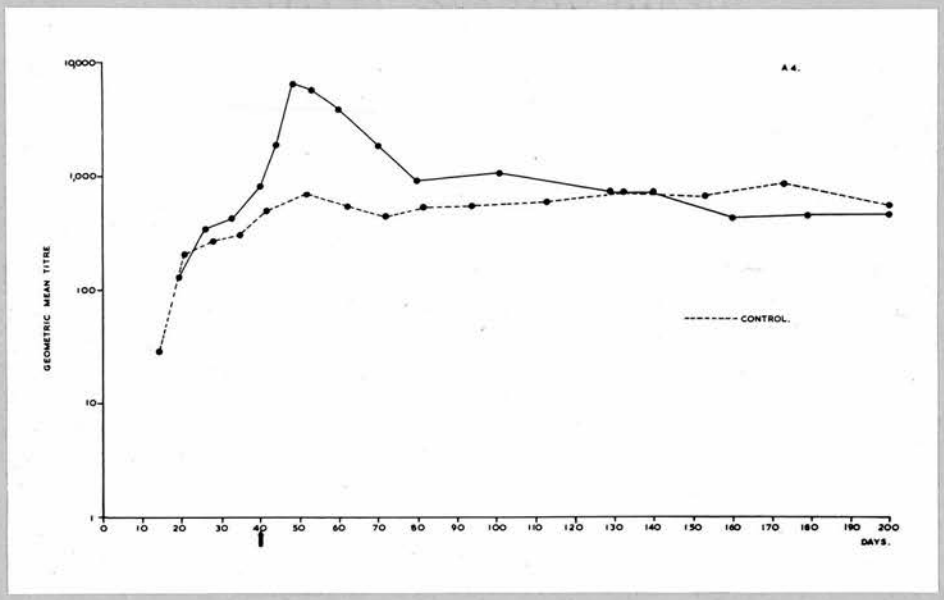
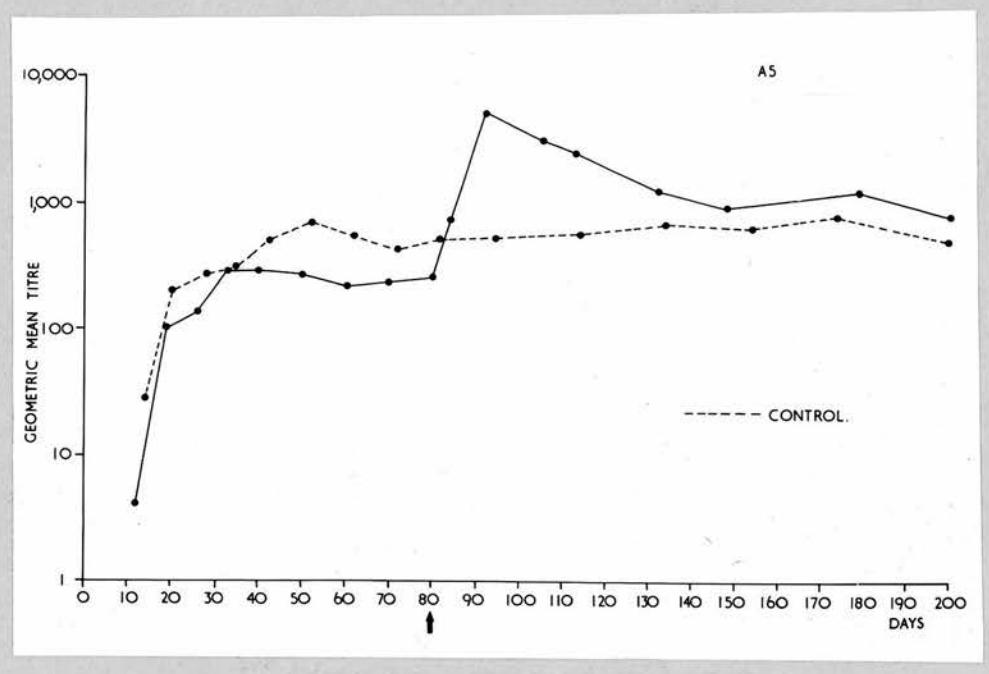




Fig.46. Antibody response curves of groups of mice given a secondary injection of 1 mg of ovalbumin in saline twenty days after (above) and forty days after (below) being inoculated with 2 mg of ovalbumin in a water-in-oil emulsion. The broken line is the response of a control group of mice given 2 mg of ovalbumin in water-in-oil emulsion only.



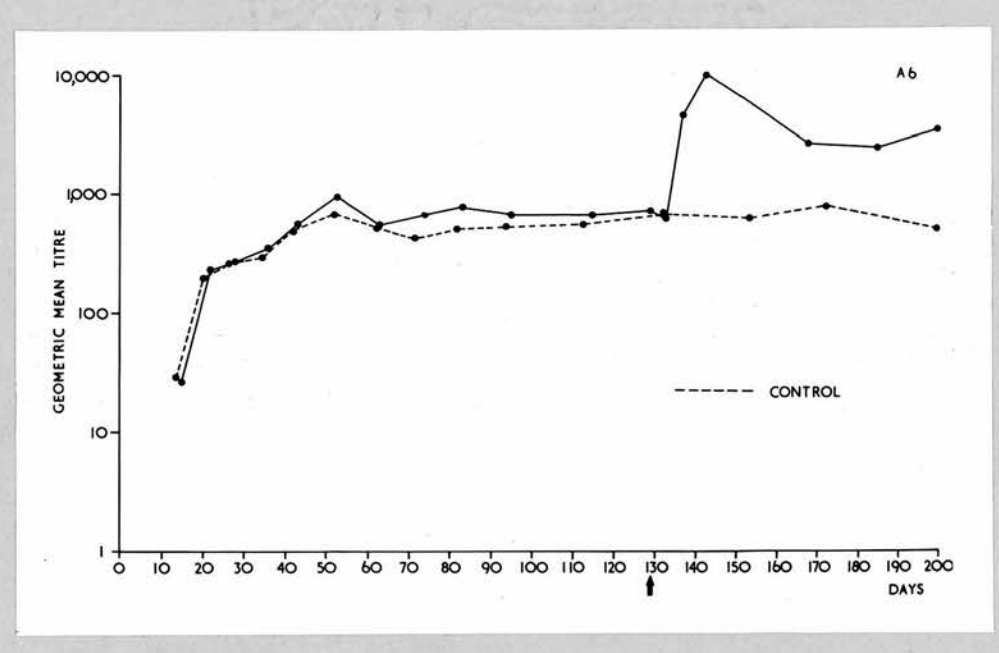




Fig.47. Antibody response curves of groups of mice given a secondary injection of 1 mg of ovalbumin in saline eighty days after (above) and one-hundred-and-twenty-nine days after (below) an inoculation of 2 mg of ovalbumin in a water-in-oil emulsion. The broken line is the response of a control group of mice given 2 mg of ovalbumin in a water-in-oil emulsion only.

Table 31. Geometric mean titres of groups of mice given a single ——— secondary injection of ovalbumin in saline at various times after an initial inoculation with ovalbumin in water-in-oil emulsion.

| Group | Number of mice (Grey) | Mean weight | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A Control | 10 | 24.2 g | Days: | | 14 | 21 | 28 | 35 | 42 | 52 | 62 | 72 | 82 | 94 | 113 | 133 | 153 | 173 | 200 | | | | |
| | | | Titre: | | 27.6 | 196 | 260 | 296 | 484 | 689 | 520 | 422 | 508 | 520 | 557 | 691 | 639 | 806 | 548 | | | | |
| B | 10 | 24.6 g | Days: | 5 | 14 | 21 | 28 | 35 | 42 | 52 | 62 | 72 | 82 | 94 | 113 | 133 | 153 | 173 | 200 | | | | |
| | | | Titre: | S | 5.6 | 76.4 | 197 | 202 | 273 | 345 | 452 | 452 | 492 | 538 | 570 | 587 | 587 | 781 | 830 | | | | |
| C | 10 | 24.0 g | Days: | 10 | 13 | 20 | 27 | 34 | 41 | 49 | 59 | 69 | 79 | 92 | 110 | 130 | 150 | 170 | 200 | | | | |
| | | | Titre: | S | 8.6 | 197 | 197 | 211 | 235 | 274 | 274 | 374 | 182 | 169 | 452 | 293 | 254 | 320 | 293 | | | | |
| D | 10 | 23.8 g | Days: | | 13 | 20 | 20 | 24 | 28 | 32 | 41 | 59 | 69 | 79 | 92 | 110 | 130 | 150 | 170 | 200 | | | |
| | | | Titre: | | 4.2 | 62 | S | 292 | 1852 | 1688 | 548 | 430 | 195 | 195 | 176 | 290 | 215 | 290 | 319 | 90 | | | |
| E | 10 | 23.9 g | Days: | | 12 | 19 | 26 | 33 | 40 | 40 | 44 | 48 | 53 | 60 | 70 | 80 | 101 | 129 | 140 | 160 | 179 | 200 | |
| | | | Titre: | | 0 | 121 | 343 | 422 | 788 | S | 1810 | 6450 | 5620 | 3880 | 1810 | 880 | 1016 | 691 | 691 | 403 | 430 | 430 | |
| F | 10 | 23.7 g | Days: | | 12 | 19 | 26 | 33 | 40 | 50 | 60 | 70 | 80 | 80 | 84 | 92 | 105 | 113 | 132 | 148 | 179 | 200 | |
| | | | Titre: | | 4.0 | 100 | 137 | 296 | 296 | 274 | 218 | 236 | 254 | S | 746 | 5260 | 3220 | 2560 | 1280 | 940 | 1280 | 806 | |
| G | 10 | 23.7 g | Days: | | 15 | 22 | 29 | 36 | 43 | 53 | 63 | 74 | 83 | 95 | 115 | 129 | 129 | 133 | 137 | 143 | 152 | 168 | 185 | 200 |
| | | | Titre: | | 26.6 | 226 | 278 | 342 | 557 | 970 | 557 | 686 | 788 | 686 | 686 | 735 | S | 640 | 4694 | 10,540 | 6640 | 2792 | 2554 | 3620 |

Days = days from the initial inoculation with water-in-oil emulsion.

S = secondary injection of 1 mg of ovalbumin in saline.

0 = no antibody detected.

curves one on top of the other with the origins (the point at which the secondary injection was given) coincident (Croxton, 1959). This has been done in figure 48 which shows that the secondary response is very similar no matter when the stimulus was given.

## 2. Investigation of the influence of the inoculation of a second antigen in adjuvant on mice already responding to an antigen in adjuvant.

The use of a mineral oil adjuvant causes the antibody response to an antigen to remain at a high level over a long period of time. In practical prophylaxis, it may be desirable to give inoculations of other antigens made up in the same adjuvant during this period. An experiment was therefore designed to study the influence of later injections of a different antigen in adjuvant on the existing antibody response of animals to another antigen which had also been given in the same adjuvant.

A water-in-oil emulsion containing 2 mg of ovalbumin per 0.2 ml was prepared by the syringe method. Mice in three groups of ten were each given 0.2 ml of the emulsion subcutaneously. A fourth group was left uninoculated.

On the 16th day, a water-in-oil emulsion

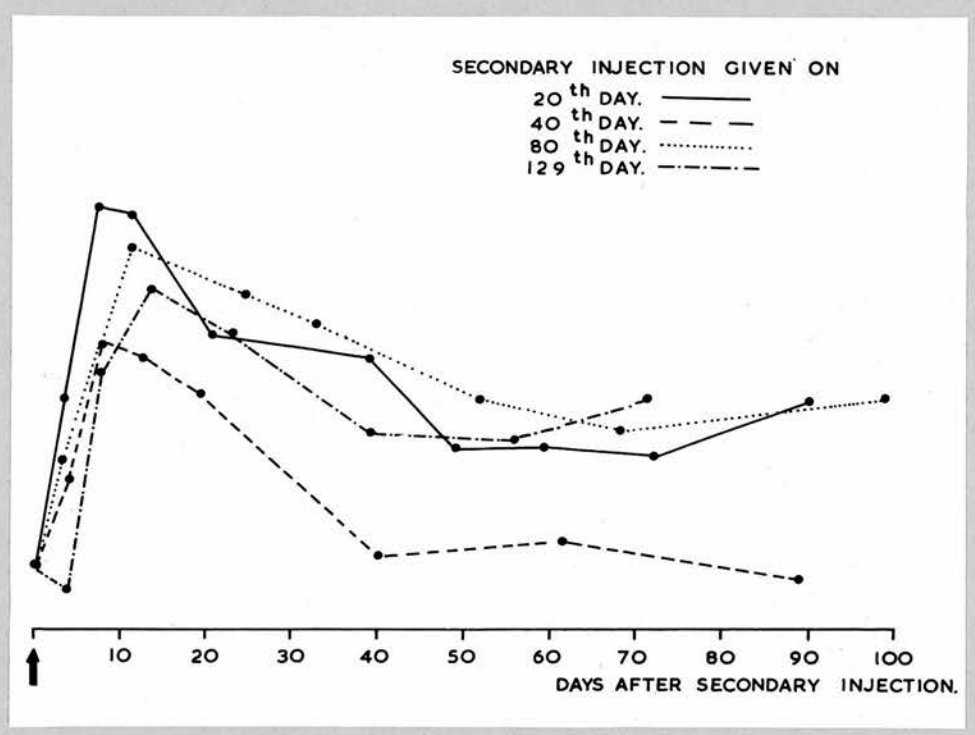



Fig.48. The secondary antibody response curves of groups of mice given 1 mg of ovalbumin in saline on the days shown above, after a primary inoculation of 2 mg of ovalbumin in a water-in-oil emulsion. These are tracings (with the titre at the time of secondary stimulation as a common origin) from the presentation of the results on semi-logarithmic axes in figures 46 and 47.

containing 2 mg of human-gamma-globulin in each 0.2 ml was similarly prepared. The uninoculated mice were each given 0.2 ml of this emulsion subcutaneously as also were the mice in one of the ovalbumin groups. In these latter, the second inoculation was made on the opposite side of the back to the site of the first inoculation.

Similarly on the 81st day, the mice in another of the groups given ovalbumin were each given 2 mg of human-gamma-globulin in 0.2 ml of water-in-oil emulsion. This emulsion had been freshly prepared and no individual control group was set up for it.

The viscosities of the two human-gamma-globulin containing emulsions were measured by the method of Berlin (1960) immediately after their preparation. The first emulsion gave a mean flow time of 18 seconds, and the second of 36 seconds, for 0.2 ml (arithmetic means of seven runs in each case).

Serum samples were collected at intervals from all of the mice and these were titrated for anti-ovalbumin and/or anti-human-gamma-globulin antibodies as required. For the anti-human-gamma-globulin antibodies, formalinized cells coated with human-gamma-globulin were used. These had been prepared and stored in exactly the

same way as those coated with ovalbumin. The results are given in table 32 and figures 49, 50, 51 and 52.

The two inoculations appear to have had no influence upon each other. There is some divergence of the antibody response curve for the second batch of human-gamma-globulin containing emulsion, from that for the first batch, perhaps due to their differing viscosities (fig. 52). Student's *t* tests to compare the means of the anti-human-gamma-globulin titres of groups C and D showed no significant difference.

| Group C | Group D | P |
|---|---|---|
| Day 68 with | Day 65 | 0.3 - 0.2 |
| Day 92 with | Day 95 | 0.4 - 0.3 |

At the end of the experiment the depot sites were examined. In the majority of mice, only one or two large depots were found, depending on whether or not the mice had received both inocula. There was little reaction visible and only slight encapsulation. This latter was, however, sufficient to allow depots to be dissected out whole. Most depots showed stratification of the emulsion as shown in figure 72.

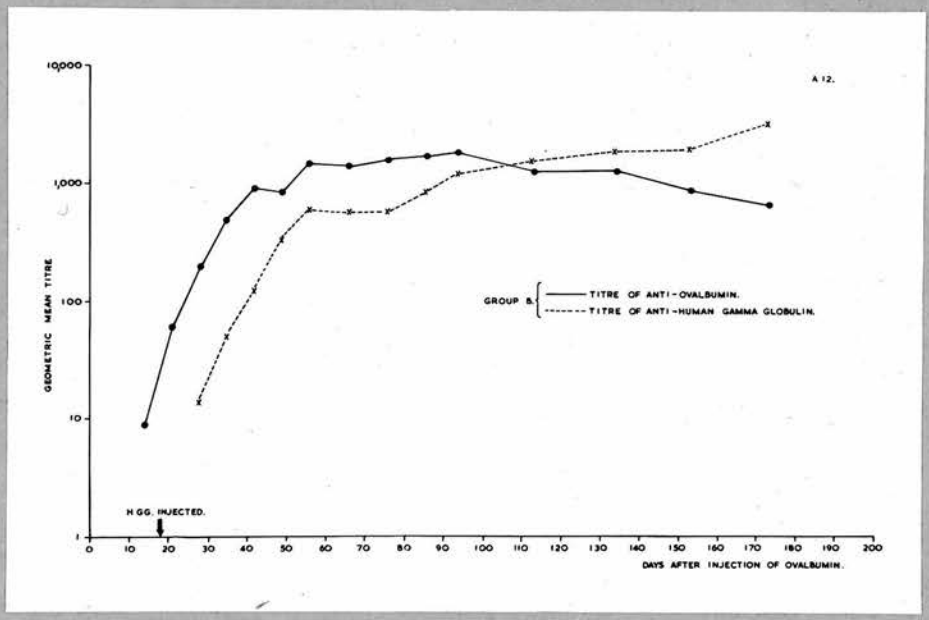



Fig.49. The antibody responses of a group of mice inoculated with ovalbumin in a water-in-oil emulsion, followed by human-gamma-globulin, also in a water-in-oil emulsion, eighteen days later.

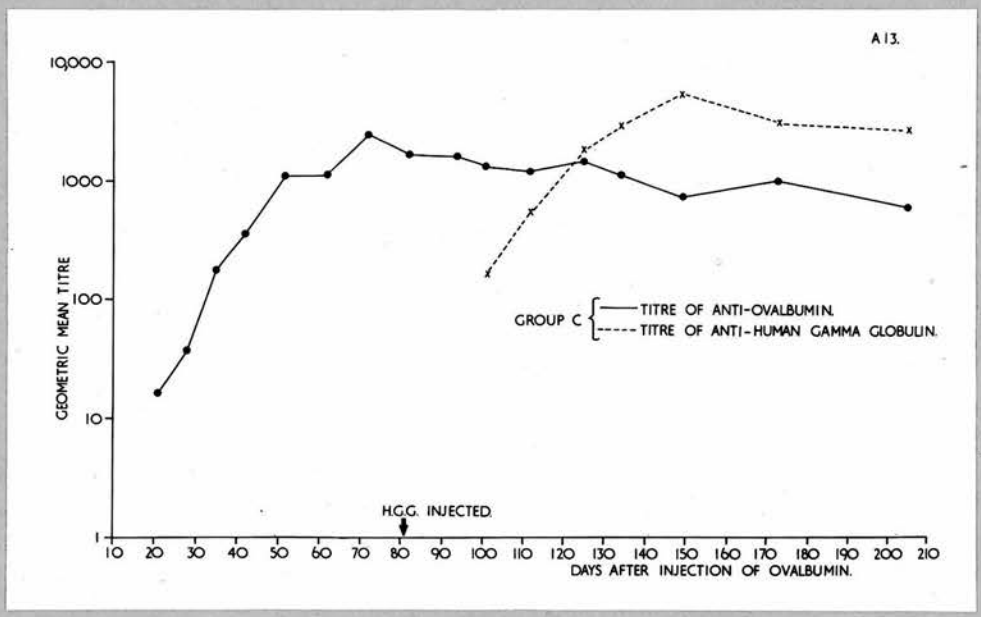



Fig.50. The antibody responses of a group of mice inoculated with ovalbumin in a water-in-oil emulsion, followed by human-gamma-globulin, also in a water-in-oil emulsion, eighty-one days later.

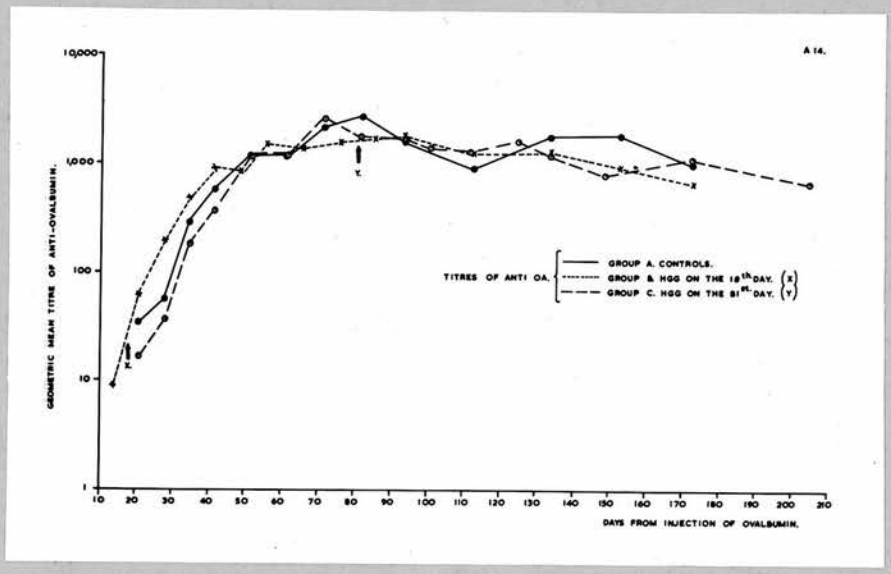



Fig.51. The anti-ovalbumin response curves of three groups of mice each of which was inoculated with 2 mg of ovalbumin in a water-in-oil emulsion. Group B was subsequently given an inoculation of human-gamma-globulin in water-in-oil emulsion on day 18 (at the arrow marked X). Group C was inoculated similarly on day 81 (at the arrow marked Y).

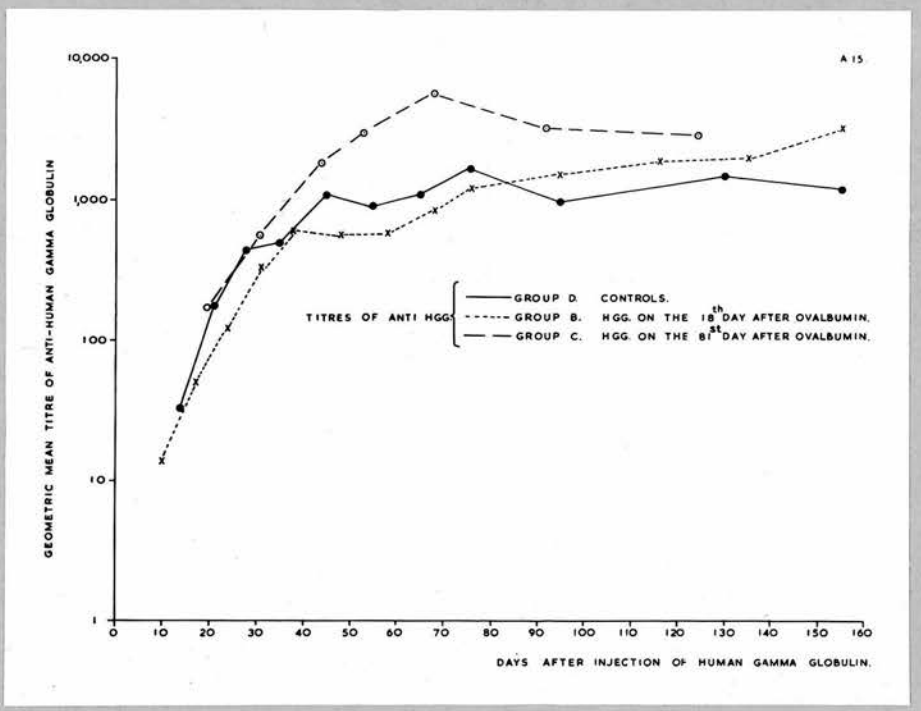



Fig.52. The anti-human-gamma-globulin response curves of groups of mice inoculated with 2 mg of human-gamma-globulin in a water-in-oil emulsion whilst already responding to ovalbumin in a water-in-oil emulsion. Group D is a control group which were not given ovalbumin.

Table 32. Geometric mean titres of anti-ovalbumin or of anti-human-gamma-globulin in the sera of groups of mice given injections of these antigens in water-in-oil emulsions.

| Group | Treatment | Number of mice (Grey) | Mean weight | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| A | Ovalbumin only | 10 | 23.9 g | Days: | 0 | 14 | 28 | 35 | 42 | 52 | 62 | 72 | 82 | 94 | 113 | 134 | 153 | 173 | | |
| | | | | Anti-OA: | 0 | 34.0 | 57.3 | 295 | 558 | 1119 | 1035 | 2076 | 2613 | 1524 | 888 | 1761 | 1710 | 957 | | |
| B | Ovalbumin on day 0 | 10 | 23.8 g | Days: | 0 | 14 | 18 | 21 | 28 | 35 | 42 | 49 | 56 | 66 | 76 | 86 | 94 | 113 | 134 | 153 | 173 |
| | | | | Anti-OA: | 0 | 9.0 | ND | 60 | 195 | 480 | 894 | 822 | 1479 | 1356 | 1524 | 1647 | 1779 | 1209 | 1245 | 870 | 645 |
| | Human-gamma-globulin on day 18 | | | Days: | - | - | 0 | 3 | 10 | 17 | 24 | 31 | 38 | 48 | 58 | 68 | 76 | 95 | 116 | 135 | 155 |
| | | | | Anti-HGG: | | | H | ND | 13.5 | 49.5 | 121 | 320 | 596 | 556 | 568 | 808 | 1184 | 1492 | 1808 | 1900 | 3620 |
| C | Ovalbumin on day 0 | 10 | 23.4 g | Days: | 0 | 21 | 28 | 35 | 42 | 52 | 62 | 72 | 81 | 82 | 94 | 101 | 112 | 125 | 134 | 149 | 173 | 205 |
| | | | | Anti-OA: | 0 | 16.5 | 37.8 | 180 | 363 | 1101 | 1182 | 2532 | ND | 1671 | 1671 | 1356 | 1266 | 1524 | 1119 | 762 | 1047 | 603 |
| | Human-gamma-globulin on day 81 | | | Days: | - | - | - | - | - | - | - | - | 0 | 1 | 13 | 20 | 31 | 44 | 53 | 68 | 92 | 124 |
| | | | | Anti-HGG: | | | | | | | | | H | ND | ND | 168 | 520 | 1880 | 2984 | 5528 | 3120 | 2870 |
| D | Human-gamma-globulin only | 10 | 23.3 g | Days: | - | - | 0 | 14 | 21 | 28 | 35 | 45 | 55 | 65 | 76 | 95 | 135 | 155 | | | | |
| | | | | Anti-HGG: | | | H | 32.5 | 171 | 422 | 485 | 1064 | 884 | 1072 | 1652 | 948 | 1436 | 1156 | | | | |

ND = not done. H = Human-gamma-globulin in water-in-oil emulsion—injected.

Days = in each case these are counted from the day on which either—injection was made. They are consistent within the groups but not from group to group as set out on this table.

HGG = Human-gamma-globulin.

## C. Investigation of the effect of alterations in the form of the emulsion



### 1. The preparation of multiple emulsions

It is probable that small particles of any water-in-oil emulsion injected subcutaneously break away from the main mass and become widely distributed in the body soon after injection (Freund and Lipton, 1955).

To encourage this process an attempt was made to supply the water-in-oil emulsion in the form of many small droplets instead of a single depot.

a) Preliminary experiments

A water-in-oil emulsion containing only saline was prepared as described in the Methods section. The viscosity of this emulsion was increased by cycling it between two syringes connected by a double hubbed needle (Berlin and McKinney, 1958).

One millilitre of the resulting emulsion was added to 9 ml of saline. Vigorous shaking broke the emulsion into small droplets but they rose to the top of the mixture very rapidly and coalesced.

The degree of dispersion was increased by ultrasonicating the mixture with the M.S.E. probe but the tiny droplets produced coalesced again within a few minutes. The addition of a small quantity of agar to the mixture did not help.

b) First multiple emulsion

Oil-in-water emulsions can readily be made either by employing a suitable emulsifier of the detergent type which promotes this type of emulsion, or in the more classical way with the vegetable gums. An attempt was made to re-emulsify the water-in-oil emulsion using one of these materials.

Two drops of 'Polywog' brand office gum were

added to the mixture of water-in-oil emulsion used above and the whole was re-treated with the ultrasonic probe. An apparently stable emulsion consisting of tiny drops of water-in-oil emulsion was formed. The drops did not coalesce or rise rapidly to the top of the liquid. This type of emulsion is termed 'multiple' (Sumner, 1954) and is shown diagrammatically in figure 53. The nomenclature given is that agreed to in discussions with the staff of the National Research Development Corporation.

c) Multiple emulsion prepared with acacia

After obtaining the promising results above, a second emulsion was prepared by the classical method of pharmacy.

Two millilitres of a water-in-oil emulsion containing only saline, were added to 0.25 g of Acacia B.P. and the mixture ground up in a mortar. Portions of saline were then added to a total of 13 ml, grinding and mixing being continued between each addition.

The dispersion of the water-in-oil phase was not as great as had been hoped so the whole was ultrasonicated with the M.S.E. probe. A cream-like mobile fluid was formed. On microscopic examination this was found to consist of tiny

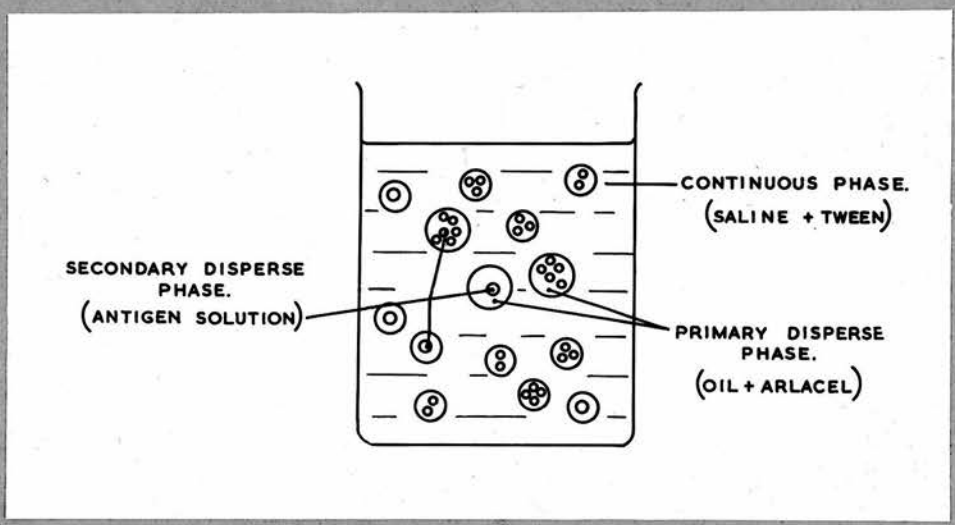



Fig.53. Diagrammatic representation of a multiple emulsion.

droplets of oil containing within themselves further minute drops. They showed no tendency to coalesce.

This multiple emulsion was tested by subcutaneous injection in two groups of five mice. One group was given the emulsion neat, and the other group was given it after dilution five times with saline, the total volume in each case was the same. Animals from each group were killed with ether and examined on the 3rd, 7th and 14th days after inoculation. Table 33 gives the results of these examinations. There was little inflammatory or fibrous tissue reaction in most cases and the depots formed were rather consolidated lumps with a cheese-like consistency. The emulsion appeared to retain its multiple form.

d) Multiple emulsion prepared with Tween 80

It was found that a stable multiple emulsion could be produced more easily by employing Tween 80 as the emulsifier rather than gum. The primary emulsion was prepared by adding 1 ml of Incomplete Adjuvant (Difco) to 1 ml of saline and ultrasonicating the mixture with the M.S.E. probe. The emulsion formed was checked to ensure that it was of the correct form and then 4 ml of saline

Table 33. Post-mortem appearances of the depots formed in mice by the injection of a multiple emulsion prepared with acacia.

| Identification number of mouse | Day on which killed | Size of depot (mm) | Type of depot (Macroscopic) | Contents of depot (Microscopic) | Inflammatory or fibrous reaction |
|---|---|---|---|---|---|
| a) Mice given neat emulsion | | | | | |
| 188 | 3 | 5 x 5 x 4 | Thick and cheesy | Multiple | None |
| 193 | 3 | 5 x 15 x 1 | Do. | Do. | Do. |
| 190 | 7 | 10 x 4 x 4 | Consolidated | Do. | Do. |
| 209 | 7 | 8 x 5 x 2 | Do. | Mostly plain oil drops | Slight inflammation |
| 207 | 14 | Not noted | Do. | Multiple | Marked, fibrous |
| b) Mice given the emulsion diluted 1:5 | | | | | |
| 189 | 3 | 4 x 3 x 1 | Thick and cheesy | Multiple | None |
| 192 | 3 | 4 x 5 x 1 | Do. | Do. | Do. |
| 191 | 7 | Many tiny dispersed drops | Not noted | Mostly plain oil drops | Slight inflammation |
| 194 | 7 | 6 x 3 x 0.5 | Consolidated | Multiple | None |
| 208 | 14 | Not noted | Do. | Do. | Do. |

Notes: The first two figures given under 'size of depot' indicate the area on the subcutaneous surface of the skin. The third figure indicates its apparent thickness.

'Plain oil drops' means that the creamy appearance of the normal water-in-oil emulsion was absent.

and 0.15 ml of Tween 80 were added to it. The whole was shaken up and ultrasonicated again for about 10 seconds with ice cooling. An excellent, stable, milk-like emulsion was formed.

On examination under the 1/12th objective of a microscope samples of the emulsion were found to consist chiefly of oil drops of 2-3 $\mu$ diameter. At least 50 per cent of these contained water phase drops within them. There were a very few larger drops and innumerable minute refractive oil droplets under 1 $\mu$ diameter showing Brownian movement.

A sample of this emulsion was stored at 37$^{o}$ and found to remain stable up to 119 days after preparation. It had broken down when examined on the 271st day. No tests in mice were made with this emulsion.

2. The antibody response of mice to the inoculation of ovalbumin in a multiple emulsion

A water-in-oil emulsion was prepared by dissolving 25 mg of ovalbumin in 0.5 ml of saline, adding 0.5 ml of Incomplete Adjuvant (Difco) and ultrasonicating the mixture with the M.S.E. probe until a very thick emulsion resulted.

The multiple emulsion was formed from this

by adding 1.5 ml of saline containing 0.02 ml of Tween 80 and ultrasonicating the mixture. This was done in several short (5-10 second) bursts interspersed with shaking. The preparations were cooled with ice during both ultrasonications.

The final multiple emulsion contained 2 mg ovalbumin per 0.2 ml. Ten mice were each given an injection of this volume of emulsion, subcutaneously over the back. Thirty-one days later they were each given a secondary stimulus of 1.0 mg of ovalbumin in saline.

Serum samples were collected at intervals from the individual mice and titrated with tanned cells. The geometric means of the results of these titrations are shown in table 34. Figure 43 shows these results as a graph together with a curve for ovalbumin given in water-in-oil emulsion (Experiment A3). These two curves fall very closely together.

3. A comparison of the antibody responses of mice to antigens given in water-in-oil and multiple type emulsions

a) Using ovalbumin as the antigen

A water-in-oil emulsion containing 2 mg of ovalbumin in each 0.1 ml was prepared as described in the Methods section. Adequate

Table 34. Geometric mean titres of ten grey mice given 2 mg of ovalbumin each in a multiple emulsion.

| Mean weight of the mice | Days after first inoculation | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | 16 | 19 | 25 | 31 | 31 | 38 | 41 | 44 | 54 | 64 | 74 | 80 | 173 | 179 |
| 33.5 g | 0 | 20 | 61 | 260 | 343 | S | 3626 | 3310 | 2712 | 1403 | 1009 | 522 | 550 | 348 | 246 |

S = injection of 1.0 mg ovalbumin in saline.

dispersion of the saline phase was obtained by re-cycling the emulsion through the syringe only. Ten mice were each inoculated subcutaneously with 0.1 ml of this material.

A multiple emulsion was formed by adding 2 ml of the above emulsion to 2 ml of saline containing 0.05 ml of Tween 80. After vigorous shaking the mixture was ultrasonicated with ice cooling for 10 seconds until a milky liquid had formed. A drop dispersed readily in water and on microscopic examination it was found to be a good multiple emulsion. Each of ten mice were inoculated with 0.2 ml of this multiple emulsion, subcutaneously.

Serum samples were collected from both groups of mice over a period of one year. The results of tanned cell titrations for anti-ovalbumin are given in table 35 and are shown graphically in figure 54.

Although for most of the experiment higher titres were recorded from mice given the multiple emulsion, this difference was not significant ($P = 0.6$ on day 71).

Ten other mice, in two groups, were also inoculated with both types of emulsion. These were killed at intervals up to the 21st day and the inoculation sites examined. Table 36 gives

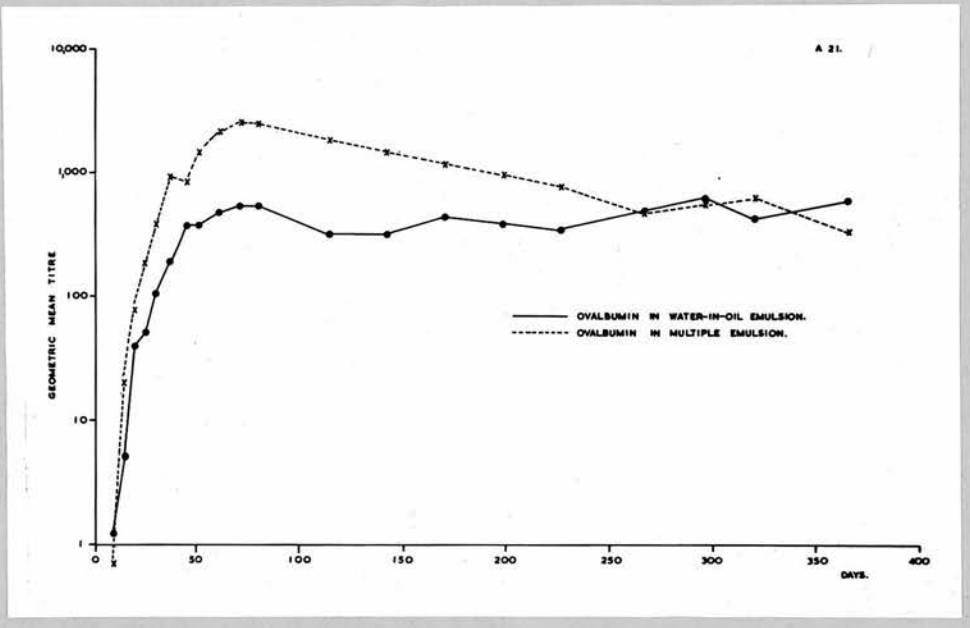



Fig.54. The antibody response curves of mice inoculated with 2 mg of ovalbumin either in a water-in-oil emulsion or a multiple emulsion.

Table 35. Geometric mean antibody titres of groups of mice inoculated with 2 mg of ovalbumin in either a water-in-oil or a multiple emulsion.

| Group | Number of mice (Grey) | Mean weight | Days after first inoculation | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 10 | 15 | 20 | 25 | 30 | 37 | 45 | 51 | 61 | 71 | 80 | 114 | 142 | 170 | 198 | 226 | 266 | 296 | 320 | 365 | 538 |
| Water-in-oil | 10 | 25.8 g | 0 | 1.2 | 5.0 | 40 | 51 | 104 | 194 | 375 | 375 | 468 | 522 | 522 | 308 | 312 | 432 | 388 | 348 | 484 | 602 | 404 | 602 | (538) |
| Multiple emulsion | 10 | 24.0 g | 0 | 0.7 | 20 | 76 | 184 | 375 | 904 | 810 | 1403 | 2177 | 2430 | 2430 | 1808 | 1451 | 1165 | 935 | 750 | 478 | 540 | 620 | 332 | 349 |

Notes: a) At day 266 two mice in the water-in-oil group appeared to show a natural secondary response (see the individual titres in table 50). The titres of these mice are included in the means calculated above.

b) The mean for the water-in-oil group on day 538 has been put in brackets to indicate that some of these mice had been interfered with, as detailed in experiment D9 , 204 days previously.

Table 36. Results of the post-mortem examination of depot sites of mice inoculated subcutaneously with water-in-oil or multiple emulsion up to 21 days previously.

| Mouse number | Day on which killed | Size of depot in mm (area) | Type of depot (Macroscopic) | Contents of depot (Microscopic) | Reactions |
|---|---|---|---|---|---|
| a) Mice given water-in-oil emulsion | | | | | |
| 150 | 1 | NR | Single | W/O | None |
| 156 | 2 | 9 x 8 | Several large drops | W/O | Some oedema |
| 152 | 3 | NR | Single | W/O | None |
| 157 | 5 | 20 x 10 | Dispersed | W/O | Slight inflammation |
| 155 | 9 | 8 x 6 | Single | W/O | Slight inflammation |
| 160 | 21 | 20 x 10 | Dispersed | NR | Slight inflammation and fibrous encapsulation |
| b) Mice given multiple emulsion | | | | | |
| 158 | 1 | NR | Single | Multiple | None |
| 151 | 2 | 7 x 10 | Single | W/O | Some oedema |
| 159 | 3 | 10 x 10 | Single | Multiple with some large W/O drops | Some oedema |
| 153 | 5 | 8 x 8 | Single | Do. | Slight oedema |
| 154 | 9 | NR | Diffuse | W/O | Extensive inflammation |
| 161 | 21 | 10 x 10 | Diffuse | W/O | Slight inflammation |

NR = not recorded. W/O = water-in-oil. Dispersed = a number of quite large drops.

Diffuse = very many tiny specks of emulsion. Single = all depot material at one site.

Note: Some of the inflammation recorded for the later animals may have been due to the handling which these animals received whilst a blood sample was being collected from them prior to destruction.

the results of these examinations.

At the end of the experiment the mice in the main groups were destroyed on the 538th day. The results of post-mortem examinations of these mice are given in table 37. At this time eight mice in each group were surviving, but the results for only four of the water-in-oil group are included in the table as the others had been used in experiment D9.

There was little difference between the type of depot formed in each case but it must be pointed out that the mice with the most discrete water-in-oil depots had been used in experiment D9 and are not reported in this series.

b) Using influenza virus vaccine as the antigen

Water-in-oil and multiple emulsion vaccines containing 'Munevan' influenza virus vaccine (Evans Medical) were prepared in such a way that the same amount of antigen was delivered in a 0.2 ml dose of either vaccine.

'Munevan' is a saline type vaccine containing three formalin killed influenza virus strains. These are 7500 HA units per ml of A2/Singapore/1/57, 2500 HA units of A2/England/1/61, and 5000 HA units of B/England/939/59. Following the work of Berlin

Table 37. Results of the post-mortem examination of the depot sites of mice killed on the 538th day after inoculation with water-in-oil or multiple emulsion adjuvant.

| Mouse number | Serum anti-ovalbumin titre on the day killed | Size of depot (Diameter in mm) | Type of depot (Macroscopic) | Contents of depot (Microscopic and/or macroscopic) | Inflammatory reaction |
|---|---|---|---|---|---|
| a) Mice given water-in-oil emulsion | | | | | |
| 131 | 1280 | NR | Many small drops grouped together in fibrous tissue | NE | None |
| 133 | 5120 | NR | Only a few tiny specks of oil seen | NE | None |
| 136 | 320 | 5 mm | Single drop. Stratified | W/O | None |
| 139 | 2560 | 2 mm (drop) | One large drop and many tiny oil specks in fibrous tissue | Oil only | None |
| b) Mice given multiple emulsion | | | | | |
| 140 | 1280 | NR | Diffuse | NE | None |
| 141 | 160 | 4 mm | Single drop and some small specks | Creamy W/O | None |
| 144 | 1280 | NR | Single | Creamy W/O | None |
| 145 | 80 | Drops up to 3 mm | A nest of small drops in fibrous tissue | Oil only | None |
| 146 | 640 | 5 mm | Single | Creamy W/O | None |
| 147 | 2560 | 5 mm | Single | Creamy W/O | None |
| 148 | 20 | NR | Diffuse, tiny drops | Oil only | None |
| 149 | 320 | Drops up to 2 mm | A nest of small drops in fibrous tissue | Oil only | None |

NR = not recorded. NE = not examined. Stratified = clear oil over a creamy layer as in figure 72. Diffuse = very many tiny specks of emulsion over a wide area.
W/O = water-in-oil. Single = all depot material in one place.

(1960) the vaccines were diluted so that each mouse received 75 HA units of strain A2/Singapore/1/57.

The two water-in-oil emulsions, one to be used as it stood and the other to be made into a multiple emulsion, were prepared as described in the Methods section. They were then each subjected to two short bursts (3-5 seconds) of ultrasound from the M.S.E. probe to increase the dispersion of the saline phase. This made them so thick that they could scarcely be poured.

The multiple emulsion was made by suspending one part of the appropriate water-in-oil emulsion (containing 'Munevan' diluted 1 in 3.3) in two parts of 1 per cent Tween 80 in saline and dispersing this by ultrasonics as described ~~under Methods~~ in a) above.

Two groups of six mice were inoculated with the water-in-oil or the multiple emulsion vaccine. Individual serum samples were collected on days 0, 21, and 35. These sera were titrated for haemagglutination inhibiting antibodies by a modification of the plastic plate method (W.H.O. 1953) using four HA units of virus.

The geometric means of the titres are given in table 38. The difference between the groups on the 35th day is just significant, P = 0.05 - 0.02.

Table 38. Geometric mean titres of haemagglutination inhibiting antibody in the sera of groups of mice given influenza virus vaccine in water-in-oil or in multiple type emulsions.

| Material inoculated | Number of mice (Grey) | Mean weight | Days after inoculation | | |
|---|---|---|---|---|---|
| | | | 0 | 21 | 35 |
| Water-in-oil | 6 | 27.6 g | 8.3 | 15.3 | 31.7 |
| Multiple emulsion | 6 | 27.2 g | 13.6 | 40.0 | 100 |

The remaining portion of the multiple emulsion was stored at 4°. This was stable for 14 weeks, but when examined after 22 weeks considerable breakdown and reversion to an oil-in-water form was found to have occurred.

The mice were destroyed with ether on the 46th day after inoculation. The results of examination of the inoculation sites are given in table 39. There was a considerable contrast between the large single depots of water-in-oil emulsion and the highly dispersed multiple emulsion.

4. The response of mice given ovalbumin in a multiple emulsion diluted with saline

To further investigate the antigenicity of an antigen contained in a multiple emulsion, mice

Table 39. Post-mortem examination of the sites of inoculation of influenza virus vaccine into mice in either water-in-oil or multiple emulsions.

| Mouse number | Type of depot seen | Type of emulsion | Inflammatory reactions |
|---|---|---|---|
| a) Water-in-oil emulsion | | | |
| 526 | Large pea-like depot, with a few smaller drops | Creamy | None |
| 529 | Do. | Creamy | None |
| 532 | Single large depot | Creamy | None |
| 536 | Numerous large drops | Creamy | None |
| 538 | Two large drops | Creamy | None |
| 541 | Three large drops | Creamy | None |
| b) Mice given multiple emulsion | | | |
| 525 | A few tiny dispersed drops | Apparently only oil | None |
| 528 | Only one tiny drop located | Do. | None |
| 531 | A few tiny drops only found with difficulty | Do. | None |
| 534 | Many small drops in fibrous tissue | Do. | Fibrous scarring |
| 540 | A few tiny drops | Do. | None |
| 543 | Numerous drops up to 1 mm diameter | Stratified appearance as in fig. 72 | None |

were inoculated with ovalbumin incorporated into an emulsion of this type which was then diluted down with saline. By this means it was hoped to obtain a greater dispersion of the droplets and at the same time some indication of the dose response relationship with this type of emulsion.

A water-in-oil emulsion containing 20 mg ovalbumin per ml was prepared as described in the Methods section.

A multiple emulsion was made from this by adding it to an equal volume of a solution of acacia and ultrasonicating the mixture with the M.S.E. probe. The mixture was cooled with ice during this procedure which was carried out in bursts of 5-10 seconds at a time interspersed with vigorous shaking.

The acacia solution was made by dissolving 0.2 g of Acacia B.P. in 2 ml saline with the aid of a 56° water bath.

Five mice were each inoculated subcutaneously with 0.2 ml of the final emulsion, equivalent to 2 mg of ovalbumin each.

A further ten mice were each inoculated with 0.2 ml of a 1:5 dilution of the emulsion in saline. This group therefore received 0.4 mg of ovalbumin each.

A third group of ten mice were each

inoculated with 0.2 ml of the original emulsion diluted 1:20 with saline, equivalent to a dose of 0.1 mg of ovalbumin each.

Sera were collected at intervals and titrated. The geometric means of the results of these titrations are given in table 40 and are shown graphically in figure 55. A typical response curve for mice given ovalbumin in water-in-oil emulsion (from table 35) is also plotted on the graph for comparison.

The difference between the water-in-oil and the neat multiple emulsion is probably significant as a Student's t test gives P = 0.05, or 1 in 20, on the 61st day. This poor response to a multiple emulsion prepared with acacia may be accounted for by the inspissated nature of the depots formed (see below).

All the three groups of mice given multiple emulsion showed a similar pattern of response. The multiple emulsion prepared with acacia was still a very effective adjuvant, but there is some indication from the graph that there was a falling off in titre after the peak, rather than the maintenance of a plateau as usually seen with water-in-oil emulsions.

The mice were destroyed with ether on the 156th day and the inoculation sites examined.

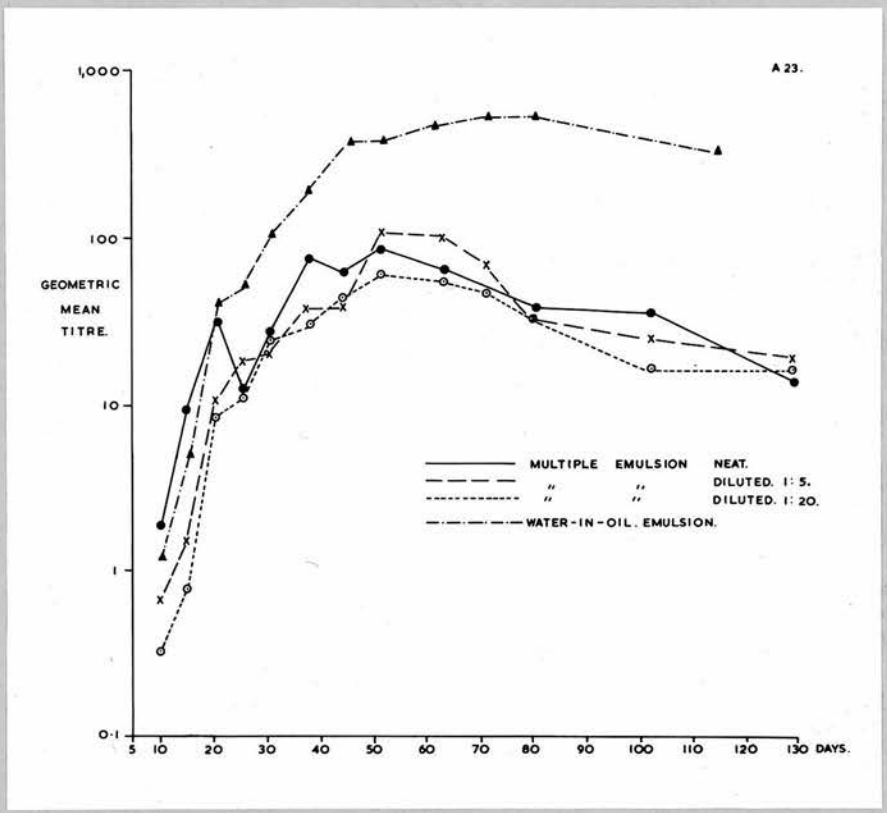



Fig.55. The antibody response of groups of mice inoculated with 2 mg of ovalbumin in a water-in-oil emulsion or in a multiple emulsion prepared with acacia. Also shown are the responses of groups of mice given the same multiple emulsion after it had been diluted 1:5 or 1:20 with saline. The mice in these latter groups therefore received either 0.4 or 0.1 mg of ovalbumin each.

Table 40. Geometric mean titres of anti-ovalbumin in the sera of groups of mice inoculated with ovalbumin in a multiple emulsion diluted with saline.

| Multiple emulsion dilution | Dose of OA per mouse | No. of mice (Grey) | Mean weight | Days after inoculation | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 10 | 15 | 20 | 25 | 30 | 37 | 44 | 51 | 63 | 71 | 80 | 102 | 129 | 156 |
| Neat | 2 mg | 5 | 30.2 g | 1.8 | 8.8 | 30.3 | 12.6 | 27.2 | 75.6 | 60.8 | 87.6 | 66.4 | ND | 38.0 | 35.0 | 13.2 | 22.8 |
| 1 in 5 | 0.4 mg | 10 | 30.4 g | 0.63 | 1.43 | 10.0 | 17.0 | 19.6 | 36.4 | 36.4 | 103 | 98.4 | 66.4 | 31.6 | 24.0 | 18.6 | 20.4 |
| 1 in 20 | 0.1 mg | 10 | 30.6 g | 0.31 | 0.74 | 8.1 | 10.8 | 23.6 | 29.6 | 42.0 | 58.0 | 52.8 | 46.8 | 30.4 | 15.3 | 15.6 | 15.2 |

ND = not done.

All had depots; these were in the form of hard, cream-coloured lumps whose size depended on the dilution of the emulsion originally injected. Under the microscope the depots showed many tiny water-in-oil droplets embedded in a hard gum matrix which was only broken up with difficulty.

Samples of the emulsions were kept at 37° and examined at intervals. The neat emulsion and that diluted 1 in 20 had broken down by the fifth month after preparation. That diluted 1 in 5 lasted until the eighth month.

5. Intravenous injection of ovalbumin in a multiple emulsion

The intravenous injection of ovalbumin in a multiple emulsion was thought to be a possible way of producing a widespread distribution of small depots throughout the body.

A water-in-oil emulsion was prepared by emulsifying 1 ml of saline containing 10 mg of ovalbumin in 1 ml of oil/Arlacel. This emulsion was prepared by the syringe method only. To the 2 ml of water-in-oil emulsion thus prepared were added 0.6 ml of Tween 80 in 8 ml saline. After shaking, the mixture was ultrasonicated with a M.S.E. probe for two periods of 20 seconds each with ice cooling. An excellent milk-like

multiple emulsion was formed.

A group of five mice were each injected with 0.2 ml of this multiple emulsion into the tail veins. They showed no discomfort after the injection. Another group of five mice were each given 0.2 ml of the emulsion subcutaneously. In each case, the mice received only 0.2 mg of ovalbumin, a tenth of the standard dose.

Sera were collected and titrated at intervals. Geometric mean titres for the two groups are shown in table 41 and in the graph figure 56. The results may also be compared with those for the intravenous injection of ovalbumin in saline alone, table 28. The pattern of response is different and the titres are much higher in the present experiment.

Though the intravenously injected multiple emulsion does show an adjuvant effect, this is confined to the speed and height of the immediate response but not its prolongation. Possibly the tiny drops in the emulsion become too dispersed in the tissues to form any true depot.

6. The antibody response of mice to ovalbumin given with an oil-in-water emulsion

The oil-in-water type of emulsion is said

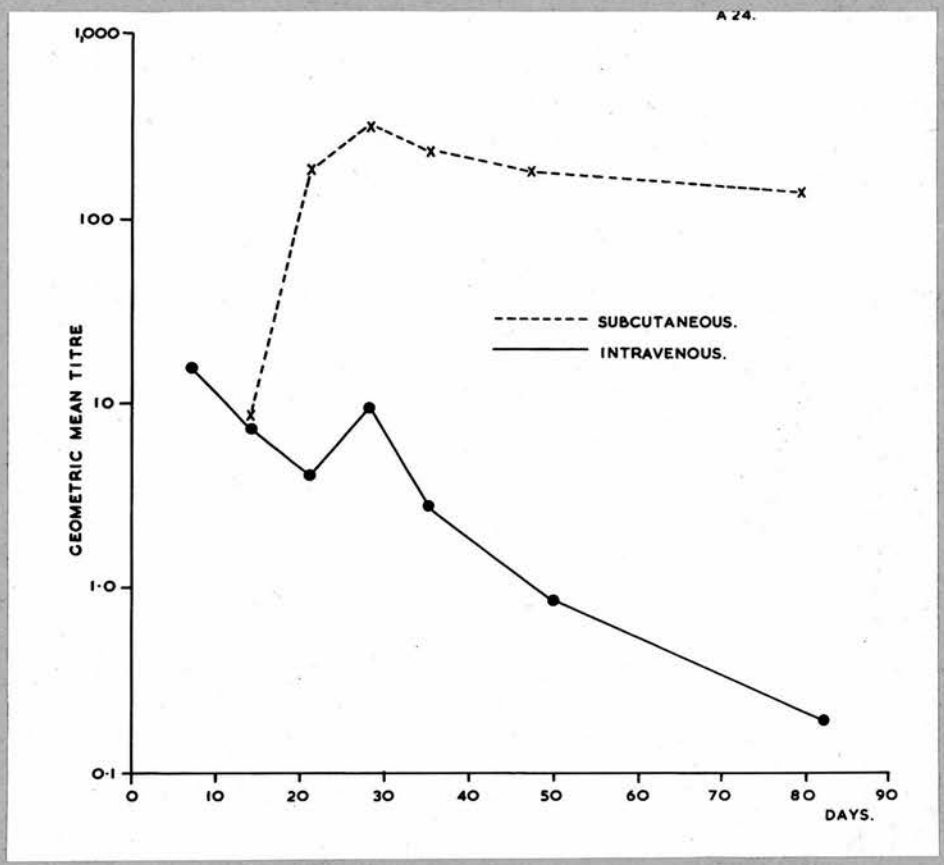



Fig.56. The antibody responses of groups of mice given 0.2 mg of ovalbumin each in a multiple emulsion, either intravenously or subcutaneously.

Table 41. Geometric mean titres of groups of mice inoculated with multiple emulsion, by either the subcutaneous or intravenous route.

| Route | No. of mice (Grey) | Days after injection | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 7 | 14 | 21 | 28 | 35 | 47 | 50 | 79 | 82 |
| Intravenous | 5 | 15.3 | 7.36 | 4.07 | 9.50 | 2.81 | ND | 0.87 | ND | 0.19 |
| Subcutaneous | 5 | ND | 8.50 | 185 | 317 | 224 | 182 | ND | 139 | ND |

ND = not done.

by several authors (Freund, 1947; Freund *et al.*, 1948; Salk, 1953) to be ineffective as an adjuvant. The first emulsion of this type studied in the present work was formed by accident and *did* show an adjuvant effect. The reality of this effect was confirmed with a second preparation made under controlled conditions.

a) This emulsion was formed in error whilst an early attempt was being made to produce a water-in-oil emulsion.

Drakeol 6-VR mineral oil, 9 parts, and Aquaphor 1 part by weight were blended together in an M.S.E. homogeniser (Aquaphor has the consistency of lanolin or motor-car grease). The mixture was then passed through a Seitz filter of the Hemming type. After centrifugation at 3000 G for 45 minutes, only a small amount of liquid had passed through and the Aquaphor had formed a layer over the upper side of the filter pad (Ford's S.B. grade).

One millilitre of the filtrate was emulsified with 1 ml of saline in which 20 mg of ovalbumin had been dissolved and Seitz filtered. The emulsification method was that of Berlin and McKinney (1958) using two syringes coupled by a

double hubbed needle. The resulting emulsion was watery and a drop allowed to fall into water dispersed immediately. The emulsion was therefore assumed to be of the oil-in-water type.

Six mice were each inoculated with 0.2 ml of this material subcutaneously over the back. Serum samples were collected at intervals up to the 31st day and titrated for anti-ovalbumin by the tanned cell test. On the 31st day all the mice were given a secondary injection of 1 mg of ovalbumin in saline. More serum samples were collected and titrated up to the 83rd day.

Table 42 gives the results of the titrations and they are shown as a graph in figure 57. This shows that there was a better response than that seen with ovalbumin in saline only (figs. 39 and 40). It was, however, not comparable with that seen when the antigen was given in a water-in-oil emulsion and the primary response was not sustained.

The mice were destroyed with ether on the 93rd day after the initial injection. Examination of the subcutaneous tissues at the inoculation site showed that small oil cysts were present in every mouse. These were found over areas of the subcutaneous tissues measuring from 20 x 15 mm to 50 x 10 mm. The cysts varied in

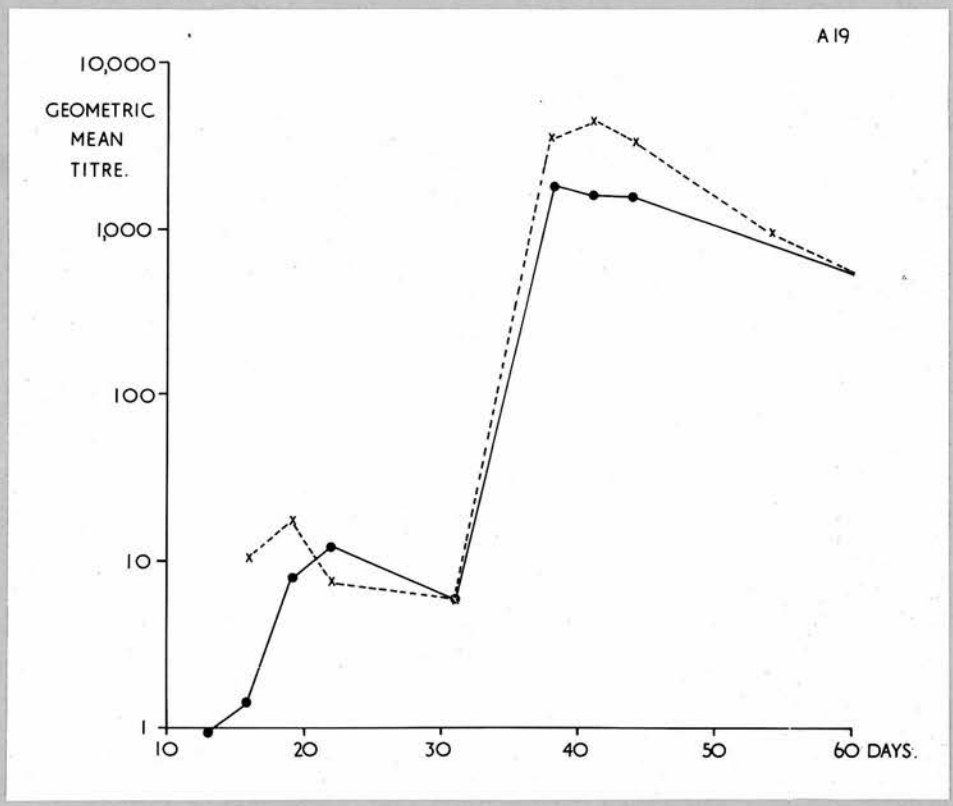



Fig.57. The antibody response curves of two groups of mice inoculated with ovalbumin in oil-in-water emulsion.

Continuous line = emulsion A, prepared with Aquaphor.
Broken line = emulsion B, prepared with Tween 80.

A secondary injection of 1 mg of ovalbumin in saline was given to each group on the 31st day.

size from a fine emulsion of tiny specks, to drops up to 3 mm in diameter. The cysts appeared to contain clear oil and there was a minimum of reaction round them.

b) The results of the first trial appeared to show that there was some stimulation of antibody production over that given by the same dose of ovalbumin in saline alone. This might, however, have been due to some of the correct type of water-in-oil emulsion having been present in the mixture. A further test was therefore devised in an attempt to eliminate this possibility.

The following materials were mixed and emulsified with the M.S.E. ultrasonic probe:

| | |
|---|---|
| Drakeol 6-VR | 1.2 ml |
| Tween 80 | 0.3 ml |
| Saline | 12.0 ml |
| Ion agar (Oxoid) | a few grains |

A milk-like stable emulsion was formed. Under the microscope this was seen to consist mainly of particles with a diameter of 1 $\mu$ or less though there were a few droplets up to 4-5 $\mu$ in diameter. There was a tendency for the particles to stick together but not to coalesce.

The emulsion remained stable when left on

the bench overnight so 24 mg of ovalbumin were dissolved in 2.4 ml of it. This was carried out in as gentle a manner as possible to eliminate any chance of the incorporation of ovalbumin into the oil drops.

Each of ten mice were inoculated subcutaneously with 0.2 ml of the emulsion. Serum samples were collected from each mouse at intervals up to the 31st day. They were then each given a secondary injection of 1 mg of ovalbumin in saline. Further samples were collected up to the 189th day. Table 42 gives the geometric mean tanned cell titres of these sera and the results are plotted on the graph figure 57. The curve closely follows that for the previous test.

The results of this test are also plotted on the graph in figure 43, together with curves for the response of mice to ovalbumin given either in saline alone or in water-in-oil emulsion. The primary response to the ovalbumin with oil-in-water emulsion lies midway between the two and the difference is significant in both cases. (Student's $\underline{t}$ test gives $P = < 0.001$ with water-in-oil emulsion and $P = < 0.001$ with saline on the 22nd day.)

The secondary response titre on the 38th day is significantly different from that to ovalbumin

in saline only (P = 0.01 - 0.001) but it is not different to that of mice given ovalbumin in water-in-oil emulsion as the primary inoculum (P = 0.2).

The mice were destroyed with ether on the 189th day after the first inoculation. No sign of any oil could be seen on macroscopic examination of the subcutaneous tissues over the back. All the other organs of the mice were quite normal.

The unused remainder of the emulsion was stored at 37°. When examined on the 68th day after preparation it was still a stable oil-in-water emulsion.

Table 42. Geometric mean titres of mice given ovalbumin in an oil-in-water emulsion.

| Emulsion used | Number of mice (Grey) | Mean weight | Days after first injection | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 10 | 13 | 16 | 19 | 22 | 31 | 31 | 38 | 41 | 44 | 54 | 64 | 74 | 80 | 83 | 189 |
| First emulsion, prepared with Aquaphor | 6 | 29.5 g | 0 | 0 | 0.92 | 1.4 | 7.6 | 12.1 | 6.0 | S | 1810 | 1613 | 1676 | ND | ND | ND | ND | 140 | ND |
| Second emulsion, prepared with Tween 80 | 10 | 29.8 g | 0 | ND | ND | 10.7 | 17.4 | 7.6 | 6.1 | S | 3620 | 4460 | 3380 | 904 | 522 | 270 | 375 | ND | 76 |

S = secondary inoculation with 1.0 mg ovalbumin in saline.

ND = not done.

## D. Investigations into the mode of action of the mineral oil adjuvants

## 1. Do the emulsifiers Arlacel A and Tween 80 have any adjuvant effect themselves?

The unexpected, short term, adjuvant effect of oil-in-water emulsion reported in experiment C6 above, could have been due to the presence of the emulsifier used to obtain a stable oil suspension. This might also account for the enhanced response to antigen given in a multiple emulsion. The emulsifier used in these experiments (Tween 80) was therefore examined to see whether it showed any adjuvant effect when used alone. Arlacel A was tested at the same time.

Three solutions were prepared each of which

contained 2 mg ovalbumin in 0.2 ml. The solvents used were:

a) Saline.

b) Five per cent Arlacel A in saline. This is equivalent to the normal concentration of Arlacel in a water-in-oil emulsion.

c) 2.5 per cent Tween 80 in saline. This is about the strength used to form the oil-in-water emulsion, but is greater than that used in multiple emulsions.

Arlacel A at this concentration did not form a true solution but a fairly rapidly separating oil-in-water type of emulsion. Solution b) was therefore shaken well to disperse the emulsifier before each injection was carried out.

Mice in groups of six were each given 0.2 ml of solutions a), b) and c) and sera were collected from them at intervals. The geometric means for titrations of the sera from each of these groups are shown in table 43 and are graphed in figure 58.

A Student's *t* test carried out on the 20th day means of the saline and Arlacel A groups gave P between 0.6 and 0.7 indicating that there was no significant difference.

Thus neither Tween nor Arlacel showed any adjuvant effect.

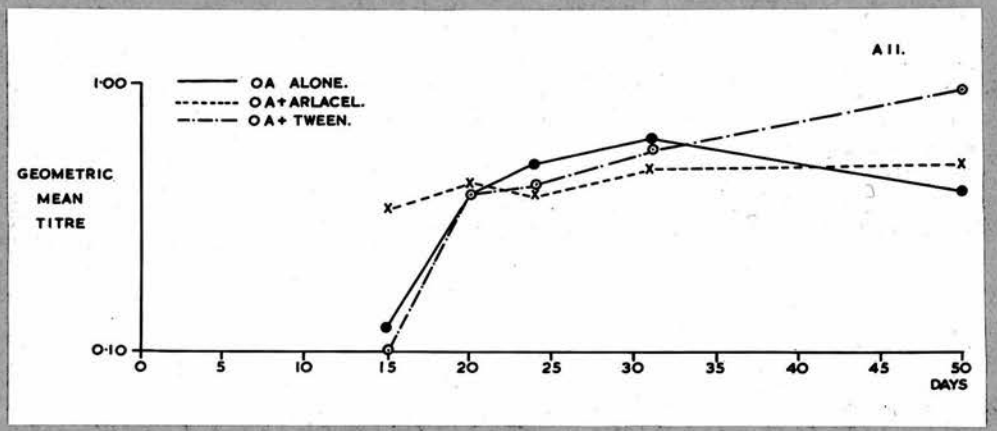



Fig.58. The antibody responses of groups of mice inoculated with 2 mg of ovalbumin in saline or with the emulsifiers Arlacel A or Tween 80.

Table 43. Geometric mean titres of mice inoculated with ovalbumin alone or in association with Tween 80 or with Arlacel A.

| Material | Number of mice (Grey) | Mean weight | Days after inoculation | | | | |
|---|---|---|---|---|---|---|---|
| | | | 15 | 20 | 24 | 31 | 50 |
| OA alone | 6 | 31 g | 0.12 | 0.38 | 0.50 | 0.62 | 0.40 |
| OA + Arlacel | 6 | 31 g | 0.34 | 0.42 | 0.38 | 0.48 | 0.50 |
| OA + Tween | 6 | 31 g | 0.10 | 0.38 | 0.42 | 0.56 | 0.96 |

2. Investigation of the influence of a saline-containing water-in-oil emulsion inoculated into mice at a different time or place to the antigen

The object of this experiment was to find out whether the water-in-oil emulsion itself or its component parts had any stimulating effect on the antibody forming mechanism when given apart from the antigen.

A water-in-oil emulsion was prepared by the syringe method. It contained saline only. Mice in six groups of five were each given 2 mg of ovalbumin dissolved in saline, on one day. Those in two of these groups had been given 0.2 ml of the water-in-oil emulsion subcutaneously one or two days previously. Another two groups

were given a similar amount of the emulsion one or two days subsequent to the ovalbumin. Of the remaining two groups, one was given the emulsion at the same time as the ovalbumin, but on the other side of the animal (interval about one minute). The other group was given the ovalbumin solution mixed with the emulsion in the same syringe. To do this, 1 ml of emulsion was taken up into the syringe, followed by 1 ml of ovalbumin solution. The mixture was shaken, moderately, before each mouse was injected, so that some of each material was delivered through the needle.

Serum samples were collected at intervals from each mouse. The geometric mean titres of the results of titration of these are given in table 44. Only group D, those mice given ovalbumin mixed in the same syringe with the emulsion, show any marked enhancement of antibody formation. The adjuvant does not therefore appear to be effective unless the antigen is contained within it. Some incorporation of antigen into the emulsion may have occurred in the case of group D.

The last column in the table gives the probability of the results for each group being different from those of mice given 2 mg of oval-

Table 44. Geometric mean titres of mice given a water-in-oil emulsion at a different time or place to the antigen.

| Group | Number of mice (White) | Mean weight | Days | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 29 | 35 | 51 | 65 | P |
| A | 5 | 27.8 g | W/O | - | OA | - | - | 0.24 | 0.29 | 0.82 | 0 | 0.3 |
| B | 5 | 28.1 g | - | W/O | OA | - | - | 1.73 | 1.73 | 4.0 | 3.0 | 0.6 |
| C | 5 | 27.8 g | - | - | W/O + OA apart | - | - | 0.76 | 2.4 | 5.5 | 3.5 | 0.5 |
| D | 5 | 27.6 g | - | - | W/O + OA mixed | - | - | 121 | 160 | 905 | 905 | ND |
| E | 5 | 28.0 g | - | - | OA | W/O | - | 0.87 | 1.37 | 1.1 | 0.45 | 0.8 |
| F | 5 | 27.9 g | - | - | OA | - | W/O | 2.7 | 4.8 | 8.3 | 6.3 | 0.3-0.2 |

W/O = water-in-oil emulsion containing saline only.
OA = 2 mg ovalbumin in saline.
ND = not done.

bumin in saline alone in experiment D1 above. This was estimated by Student's *t* test using the 35th day results of the present experiment and the 31st day results from experiment D1. None of them were significantly different. No estimate of P was made for group D.

3. The dilution of a water-in-oil emulsion with more oil

In this experiment an attempt was made to see whether an excess of the oil phase, together with a reduced antigen content, would so prevent the escape of effective quantities of antigen from the emulsion that only a poor antibody response would follow its administration.

A water-in-oil emulsion containing 2 mg ovalbumin per 0.2 ml was prepared as described in the Methods section.

A control group of five mice were each inoculated with 0.2 ml of this emulsion subcutaneously, i.e. 2 mg ovalbumin each.

A second group of ten mice were each inoculated subcutaneously with 0.2 ml of an emulsion made by diluting the original material 1:5 with more oil/Arlacel. Each mouse in this group therefore received 0.4 mg ovalbumin.

A third group of ten mice were given the

original emulsion diluted 1:20 with oil/Arlacel. Each mouse received 0.2 ml of the mixture, equivalent to 0.1 mg ovalbumin.

Sera were collected at intervals and the geometric mean titres of each group are given in table 45 and are shown graphically in figure 59. Student's t tests carried out on the 51st and 61st days means of the control and 1:5 dilution group gave P = 0.1-0.2 in both cases. The differences between the groups are, therefore, not significant, though the diluted emulsions gave the better titres.

Post-mortem examination of the sites of injection were carried out on the 271st day when the depot contents were collected for use in experiment D10. Depots were present in all cases, and were single in form or consisted of a few large drops. Most of them showed stratification, i.e. there was a clear layer of oil over creamy material as shown in figure 72.

4. To investigate the effect of incorporating ovalbumin into adjuvant materials without a water phase

The objects of these experiments were to find out whether:

(i) the presence of the water phase was necessary for adjuvance;

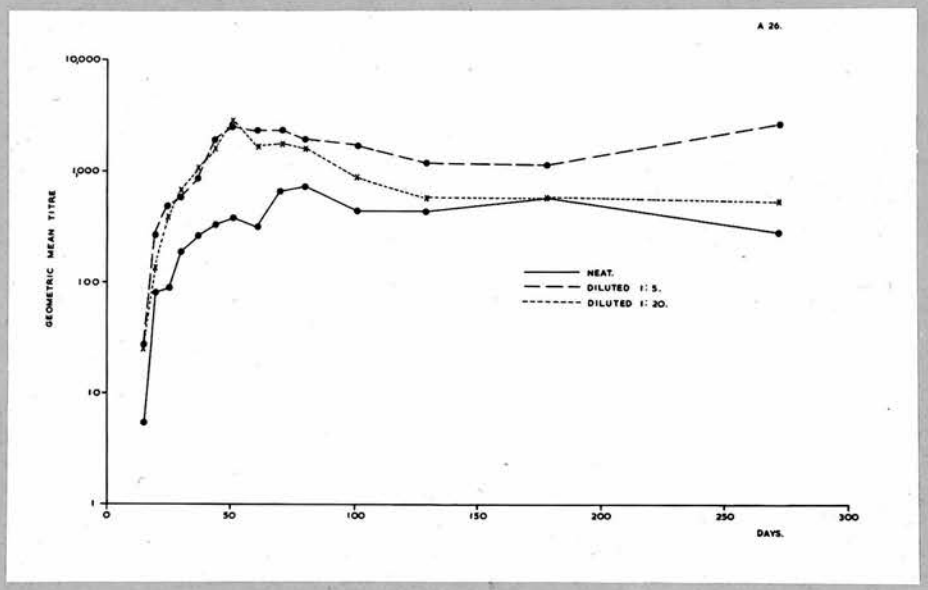



Fig.59. The antibody responses of groups of mice inoculated with 2 mg of ovalbumin in a water-in-oil emulsion, or with the same emulsion diluted 1:5 or 1:20 with more oil/Arlacel. The latter groups therefore received 0.4 or 0.1 mg of ovalbumin each.

Table 45. Geometric mean titres of groups of mice inoculated with ovalbumin in a water-in-oil emulsion, either as prepared, or diluted with more oil/Arlacel.

| Dilution of water-in-oil emulsion injected | Dose of OA per mouse | Number of mice (Grey) | Mean weight | Days after injection | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | 15 | 20 | 25 | 30 | 37 | 44 | 51 | 61 | 71 | 80 | 101 | 129 | 178 | 271 |
| Neat | 2 mg | 5 | 29.1 g | 5.6 | 80 | 88 | 184 | 252 | 313 | 352 | 303 | 632 | 696 | 410 | 410 | 540 | 254 |
| 1 in 5 | 0.4 mg | 10 | 26.1 g | 27.0 | 260 | 470 | 556 | 824 | 1872 | 2330 | 2248 | 2288 | 1870 | 1619 | 1123 | 1072 | 2560 |
| 1 in 20 | 0.1 mg | 10 | 26.6 g | 25.0 | 136 | 372 | 640 | 1004 | 1500 | 2600 | 1568 | 1652 | 1500 | 828 | 528 | 540 | 493 |

(ii) antigen could be so locked up in the oil that it was inactive;

or (iii) there was any solution or adsorption of the antigen by the adjuvant materials.

Ovalbumin was incorporated into oil or oil/Arlacel to make four preparations as follows:

a) Ovalbumin in oil

Freeze dried ovalbumin was mixed with the oil Drakeol 6-VR so that 0.1 ml contained 2 mg ovalbumin. The mixture was then treated with the M.S.E. ultrasonic probe until the antigen was well dispersed. The mixture then showed the shot-silk appearance seen when ovalbumin is being crystallized from ammonium sulphate solution.

Ten mice were each given 0.1 ml of this mixture subcutaneously. Sera were collected at intervals and titrated with preserved tanned cells. On the 66th day a secondary injection of 1 mg of ovalbumin in 0.2 ml saline was given to each mouse. Further sera were then collected at intervals.

b) Ovalbumin in oil, centrifuged

A similar mixture to the above was prepared and allowed to stand on the bench for four days being shaken occasionally. On the fourth day it

was centrifuged at 3200 G for 30 minutes. The ovalbumin then presented the appearance of a film of frosted glass over the bottom of the vial.

The supernate was carefully taken up into a syringe and three mice were each inoculated subcutaneously with 0.2 ml. Sera were collected at intervals and titrated with a sensitive batch of fresh tanned cells.

No secondary injection was given to these mice.

c) Ovalbumin in oil and Arlacel

(i) Freeze dried ovalbumin was added to the standard mixture of nine parts of Drakeol 6-VR and one part of Arlacel A, so that 0.1 ml contained 2 mg ovalbumin.

The mixture was ultrasonicated and showed the same silken swirls noted in a) above.

Ten mice were each inoculated with 0.1 ml subcutaneously and sera were collected at intervals. These sera were titrated with preserved cells.

No secondary inoculations were given to this group.

(ii) A similar batch of material was prepared and a portion of it used to inoculate five mice subcutaneously. Sera were collected and

titrated with fresh cells where the titres were low.

d) Ovalbumin in oil and Arlacel, centrifuged

The remainder of the preparation of ovalbumin suspended in oil/Arlacel from c) (ii) above was allowed to stand on the bench for four days with occasional shaking. It was then centrifuged at 3200 G for 30 minutes and the supernate inoculated subcutaneously into three mice in 0.2 ml quantities. Sera were collected at intervals and titrated with fresh cells.

The results for these experiments are combined in table 46 and in the graph figure 60. These show that there is a normal adjuvant type of response with ovalbumin in the oil/Arlacel mixture, but only a slight response if the ovalbumin is removed by centrifugation. It also appears that only enough ovalbumin can escape from plain oil to effect sensitization to a secondary injection of the antigen.

Post-mortem examination of the depots

a) Ovalbumin in oil (examined on day 219):

The depots showed single or multiple oil droplets which did not show any visible inclusions.

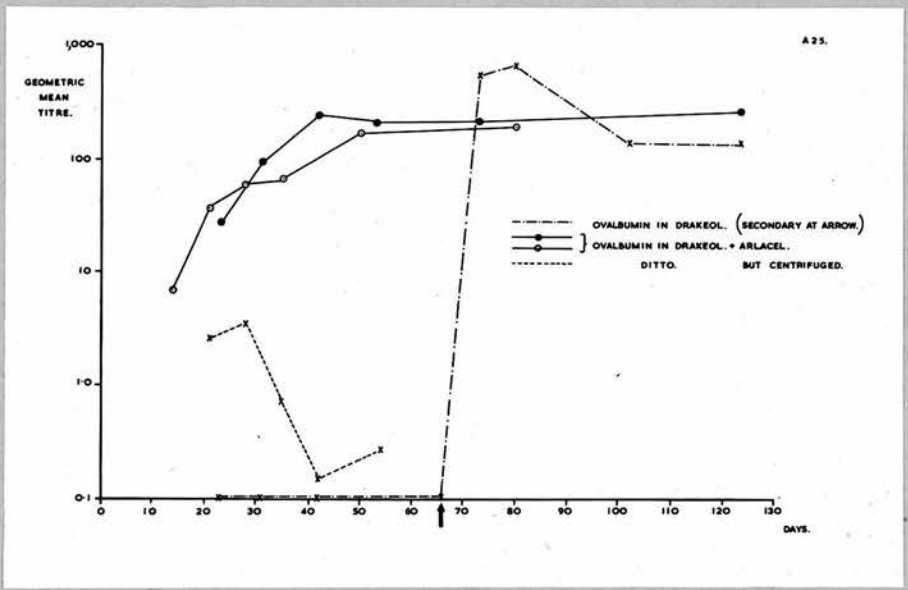



Fig.60. The antibody response curves of groups of mice inoculated with ovalbumin suspended in oil alone or in oil/Arlacel. Also their response to the latter mixture after removal of the ovalbumin by centrifugation.

b) Ovalbumin in oil, centrifuged (examined on day 86):

Only large clear oil drops in single or multiple depots were seen.

c) Ovalbumin in oil/Arlacel (i. examined on day 219; ii. examined on day 82):

(i) There was markedly more reaction and fibrous encapsulation of these depots than that seen with oil alone in a) and b). The oil drops themselves were distinctly cloudy.

(ii) Single or multiple depots were seen. Where the oil drops were large enough they appeared to contain a yellowish deposit in the lower half giving the same stratified appearance illustrated in figure 72. This material could easily be resuspended in the supernatant oil.

d) Ovalbumin in oil/Arlacel, centrifuged (examined on day 86):

The larger drops in these depots were seen to contain some fine cloudy white inclusions as in c) above. Attempts to identify the included material by microscopic examination and staining were unsuccessful.

Table 46. Geometric mean titres of groups of mice inoculated with —— ovalbumin in Drakeol 6-VR or in oil/Arlacel.

| Material | Number of mice (White) | Mean Weight | Days after initial inoculation | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 14 | 21 | 23 | 28 | 31 | 35 | 42 | 50 | 53 | 54 | 66 | 66 | 73 | 80 | 82 | 102 | 123 |
| OA in Drakeol | 10 | 27.4 g | - | - | 0 | - | 0 | - | 0 | - | - | - | 0 | S | 556 | 686 | - | 146 | 146 |
| OA in Drakeol, centrifuged | 3 | ND | 0 | 0 | - | 0 | - | 0 | 0 | - | - | 0 | - | - | - | - | - | - | - |
| OA in oil/Arlacel | (i) 10 | 25.9 g | - | - | 27.6 | - | 91.8 | - | 242 | - | 210 | - | - | - | 226 | - | - | - | 278 |
| | (ii) 5 | ND | 6.8 | 30.7 | - | 58.0 | - | 66.6 | - | 175.6 | - | - | - | - | - | - | 200 | - | - |
| OA in oil/Arlacel, centrifuged | 3 | ND | 0 | 2.57 | - | 3.4 | - | 0.70 | 0.15 | - | - | 0.27 | - | - | - | - | - | - | - |

ND = not done.

\- = no serum collected.

0 = no antibodies detected.

S = secondary injection of 1 mg of ovalbumin in saline.

## 5. Studies of the dynamics of water-in-oil emulsions

The experiments to be described were attempts to find out how easily an antigen could escape from a water-in-oil emulsion. Could it escape by merely diffusing through the oily barrier? Was break-down of the emulsion necessary for its release? Did other materials enter the emulsion whilst it was in the animal?

a) Ordinary Ochterlony double diffusion plates made with 1 per cent New Zealand agar in saline were poured in small petri dishes. Various patterns of wells were cut in them using either a Feinberg cutter (Shandon Scientific Co., London) or cork borers and a scalpel. Where a trough was cut right across the plate great care was taken to dry the glass surfaces of this with filter paper fragments before the reagents were added. Some of the patterns tried are shown in figure 61 a-d, the troughs across the plate being about 2 mm wide. Neat egg-white was placed in the wells on the left-hand side of each plate, oil/Arlacel mixture in the trough and an anti-egg-white serum (R5F) in the right-hand wells. In type A the serum was diluted 1:8 and in the others 1:2. The plates were then incubated at $37^{o}$ and inspected every day.

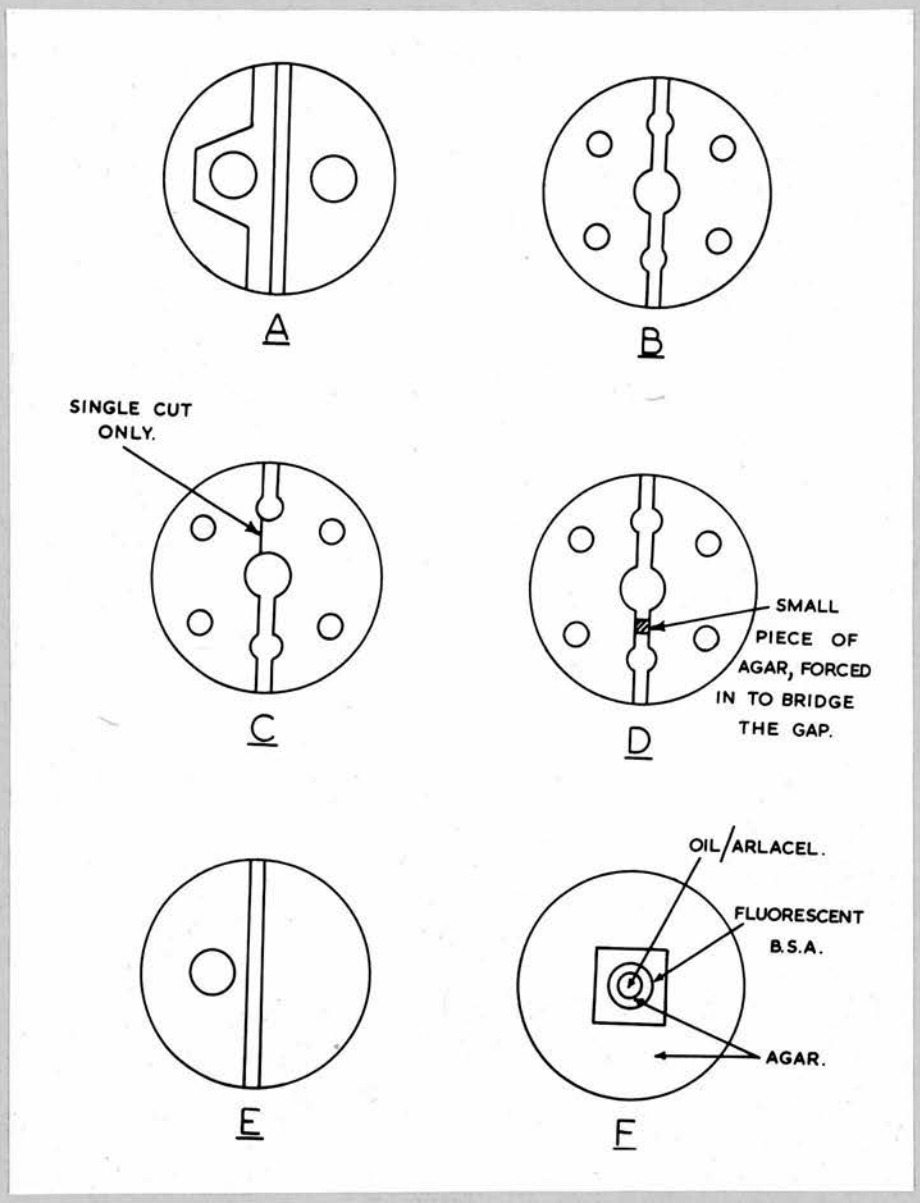



Fig.61. Patterns of wells and troughs cut in an agar layer in petri dishes. In plate A some agar has also been removed from the surroundings of the left-hand well.

In each plate overnight incubation produced cloudy smoke-like swirls of some material in the oil/Arlacel. After a few days this settled to form a deposit over the bottom of the trough. Precipitation lines appeared in plate D, and in one of three of type A but not in types B and C. Great care was taken to eliminate any moisture in the trough of plates of type A which might allow the reagents to track across it. In the case of plate type C a single knife cut seems to have been a sufficient barrier. It may be, therefore, that some antigen can escape across the oil/Arlacel barrier.

b) In an attempt to identify the cloudy material appearing in the oil/Arlacel, plates of types E and F were prepared. A fluorescein labelled (Nairn, 1962) preparation of bovine serum albumin (Armour) was placed in the well of each. The plates were incubated at $37^{o}$ and examined each day under the light from a Hanovia Laboratory U.V. lamp fitted with a Corning 5840 filter. In the E type plates, no fluorescent material was seen to cross the trough over a period of seven days. When, after this, the agar on the antigen side was pushed across to contact the plain agar on the other side,

transfer of material did occur.

Samples of the oil/Arlacel with its smoke-like deposit from the plate of type F was examined under the U.V. microscope. The contained particles could not be identified nor were any of them fluorescent.

c) Plates were prepared filled with 1 per cent New Zealand agar in saline to which a solution of bovine serum albumin (Armour) which had been fluorescently labelled (by the method of Nairn, 1962) was added whilst it was cooling. Wells were cut in this and filled with oil/Arlacel. After incubation at 37° for four days samples of the cloudy oil/Arlacel were collected and examined under the U.V. microscope. Tiny fluorescent fragments of agar were seen and also fluorescent drops of various sizes.

d) In order to find out whether antibody could enter the emulsion to combine with the antigen, water-in-oil emulsion depots from mice used in experiment B1 were examined.

These mice, which had been given a secondary injection of ovalbumin on the fifth day after receiving ovalbumin in a water-in-oil emulsion, were killed with ether on the 233rd day. Six depots were collected and the emulsion broken by

adding ether to it. A little distilled water was then added. The mixture was well shaken and allowed to stand for an hour before the antigen layer was pipetted off.

A portion of the antigen layer was examined by electrophoresis in cellulose acetate paper and an ovalbumin control was run at the same time. Figure 62 shows that the only material present appeared to be ovalbumin.

Another portion was added to an equal volume of anti-ovalbumin serum R4C and incubated overnight at 37°. When tested with tanned red cells the mixture gave a titre four-fold lower than that of a control. An unexpectedly small reduction.

e) To investigate further, a water-in-oil emulsion and its separate components were set up as Oakley-Fulthorpe type tube diffusion tests (Crowle, 1961).

The first layer placed in a precipitin tube was a mixture of equal parts of 2 per cent agar and serum, or saline as a control. The second layer was 1 per cent agar alone and the third layer the material under test. The tubes were incubated for one day at 37° and then kept for some months at 4°.

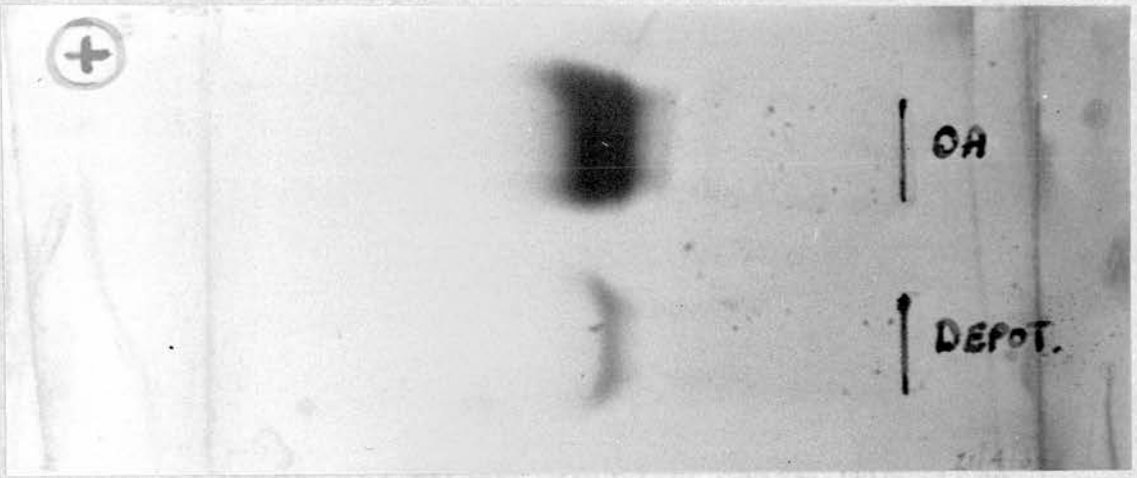



Fig.62. Electrophoresis on cellulose acetate paper.

Above: Ovalbumin control.
Below: The water phase from water-in-oil emulsion depots collected from mice 233 days after inoculation.

Electrophoresis carried out for 3 hours.

Table 47 shows how the components were arranged in the tubes and the results obtained. Precipitation lines were only seen in the tube with anti-egg-white serum in the bottom layer and ovalbumin in a water-in-oil emulsion at the top. The supernate from an attempt to dissolve ovalbumin in oil/Arlacel (experiment D4d) was inactive in this test though it had shown slight antigenic activity when inoculated into mice (fig. 60).

Both Arlacel and the oil/Arlacel mixture developed a cloudy precipitate when allowed to stand for some months. This was similar to the cloudiness in the wells of agar plates reported in a) and c) above and in the depots of oil/Arlacel, experiment D4. It presumably consisted of tiny water droplets drawn into the mixture.

## 6. A comparison of the antibody response of mice to ovalbumin given with aluminium hydroxide or with water-in-oil adjuvants

If all adjuvants of the depot forming type have the same mechanism of action, close agreement might be expected between the antibody responses to them whatever their chemical composition.

Table 47. Results of tube diffusion tests with ovalbumin in water-in-oil emulsion and its components.

| Materials in the layers | | 2 - 7 days after preparation | | Four months after preparation | |
|---|---|---|---|---|---|
| Bottom layer | Top layer | Cloudiness in top layer | Precipitation lines in the middle layer | State of top layer | State of emulsions |
| Saline | Drakeol 6-VR | None | None | Clear | - |
| Saline | Arlacel A | Yes | None | Thick white deposit over the agar surface | - |
| Saline | Oil/Arlacel | None | None | Slight white deposit over the agar surface | - |
| Normal serum | Oil/Arlacel | None | None | Thick white deposit over the agar surface | - |
| Anti-EW | Do. | None | None | Fungus growth | - |
| Saline | Water-in-oil emulsion containing saline only | None | None | Normal | No breakdown but a layer of oil present over white emulsion |
| Anti-EW | Do. | None | None | Normal | Do. |
| Saline | Water-in-oil emulsion containing ovalbumin | None | None | Breaking down | Stratified appearance as shown in figure 72 |
| Normal serum | Do. | None | None | Complete breakdown | Oil over water |
| Anti-EW | Do. | - | Yes | Do. | Do. |
| Normal serum | Supernate from oil/Arlacel/OA mixture experiment 4d | None | None | Clear | - |
| Anti-EW | Do. | None | None | Clear | - |

Normal serum = normal rabbit serum.

Ovalbumin was adsorbed on to aluminium hydroxide gel ('Alhydrogel' Dansk Svovlsyre-OG Superphosphat-Fabrik) as described in the Methods section of this study.

Ten mice were each incoulated subcutaneously with 0.2 ml of the suspension so prepared, this being equivalent to a dose of 2 mg of ovalbumin each. Another five mice were each given 2 mg of ovalbumin in 0.2 ml of the standard type of water-in-oil emulsion. This emulsion had been prepared with the aid of a 'Bel' cream maker.

At suitable intervals sera were collected from the mice and titrated with tanned cells. The geometric mean titres of the results of titrations for each of the groups are given in table 48 and are graphed in figure 63.

A statistical comparison of the difference between the means of each group on the 39th day gave P = 0.3 - 0.2 which is not significant.

Table 48. Geometric mean antibody titres of groups of mice inoculated with ovalbumin adsorbed on to aluminium hydroxide, or given in a water-in-oil emulsion.

| Group | Number of mice (Grey) | Mean weight | Days after inoculation | | | | |
|---|---|---|---|---|---|---|---|
| | | | 25 | 39 | 49 | 59 | 69 |
| Aluminium hydroxide | 10 | 32.5 g | 343 | 686 | 640 | 1374 | 1194 |
| Water-in-oil | 5 | 31.0 g | 738 | 1280 | 1470 | 3378 | 3378 |

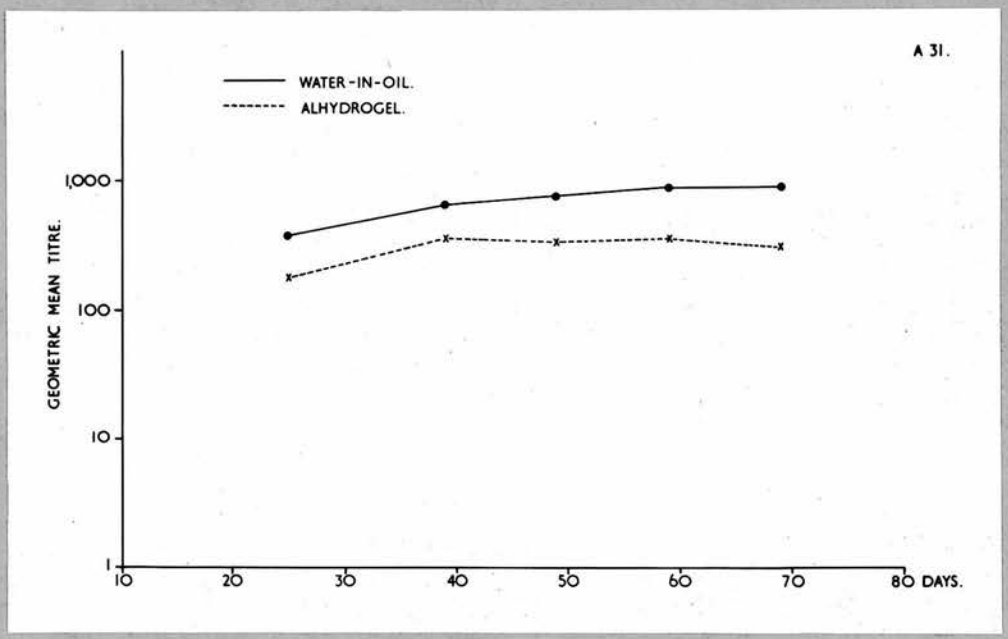



Fig.63. Antibody response curves of groups of mice inoculated with 2 mg of ovalbumin either in a water-in-oil emulsion or adsorbed on to aluminium hydroxide.

## 7. The influence of anti-ovalbumin serum given at the same time as ovalbumin in a water-in-oil emulsion

The adjuvant effect of a mineral oil emulsion could be due to an initial stimulating action of the oil or emulsifier on antibody forming cells in the presence of antigen. The administration of an anti-ovalbumin serum at the same time as an inoculation with ovalbumin in water-in-oil emulsion might block any action of this type.

Ten mice were each given a subcutaneous injection of 0.4 ml of a mouse anti-ovalbumin serum. This serum was a pool of sera from previous experiments and gave a titre of 1:2560 with tanned cells.

Fifteen minutes later these mice and also another control group of four were each given a subcutaneous injection of 2 mg of ovalbumin in a water-in-oil emulsion. The injection was made on the opposite side to the serum.

Serum samples were collected at intervals and titrated. Geometric means of the titres of the sera at each bleeding are given in table 49 and are shown graphically in figure 64. The two curves show little difference. A Student's *t* test carried out on the results for the 31st day, when the divergence was at maximum, gave P between 0.2 and 0.3 which is not significant.

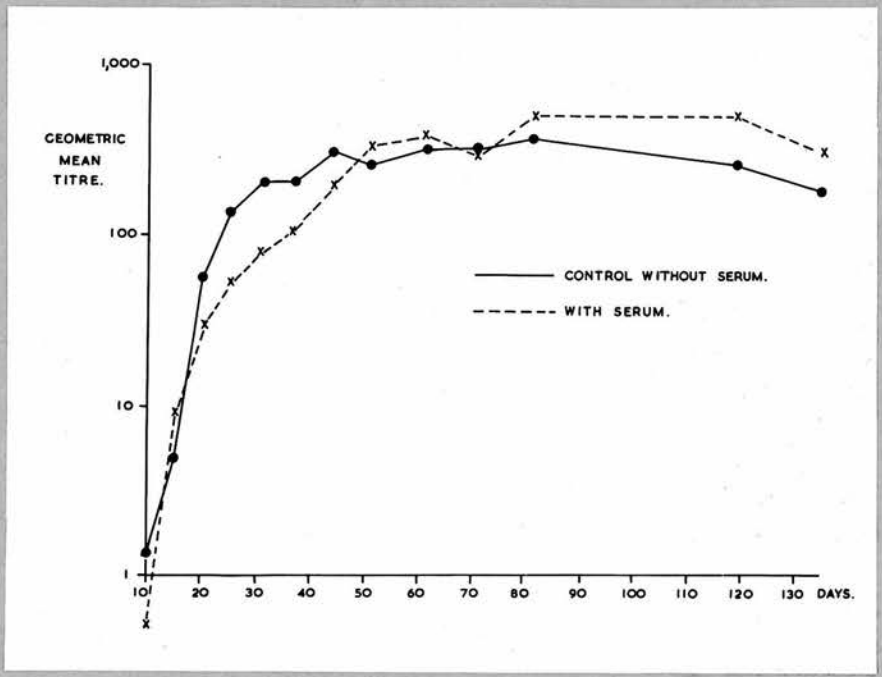



Fig.64. Antibody response curves of two groups of mice each inoculated with 2 mg of ovalbumin in water-in-oil emulsion. One group (broken line) was given an inoculation of mouse anti-ovalbumin serum 15 minutes before the antigen.

Table 49. Geometric mean titres of a group of mice given anti-ovalbumin serum before an inoculation of 2 mg ovalbumin in water-in-oil emulsion, and of a control group given no serum.

| Group | No. of mice (Grey) | Mean weight | Days after inoculation | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 10 | 15 | 20 | 25 | 31 | 37 | 44 | 51 | 61 | 71 | 81 | 119 | 135 |
| Serum | 10 | 27.7 g | 0.51 | 9.0 | 31 | 53 | 80 | 111 | 207 | 343 | 394 | 306 | 520 | 508 | 320 |
| No serum | 4 | 32.2 g | 1.2 | 5.0 | 56 | 135 | 208 | 208 | 316 | 268 | 320 | 320 | 380 | 269 | 190 |

8. Methylene blue in a water-in-oil emulsion

In an attempt to find out over how long a period materials given in a water-in-oil emulsion were able to escape from the depot, a dye which would be excreted in the urine was incorporated into an emulsion and inoculated into mice. Strauch (1929) had carried out a rather similar experiment using methylene blue emulsified in olive oil. He found that the dye was excreted over a period of several weeks.

A water-in-oil emulsion was prepared as described in the Methods section, using a 4 per cent solution of methylene blue in normal saline as the water phase.

Three white mice were each given 0.2 ml of the emulsion subcutaneously over the back. Another three white mice were each given 0.1 ml of 4 per cent methylene blue in saline only.

Each group of mice was kept in a baking tin type of mouse cage with ample food and water but no bedding except a sheet of white blotting paper. To prevent the blotting paper being chewed by the mice it was covered with a sheet of perforated zinc. Clean sheets of paper were placed in the cages every day.

For the first two days after the inoculations there was very marked staining of the paper

in each cage by the excreted dye in the urine of the mice. Thereafter this became much lighter each day. It was undetectable by the 12th day in the cage of the mice given the dye in saline, but did not disappear from the cage of those given the dye in water-in-oil emulsion until the 19th day.

9. The reinjection of depot contents into the mice from which it was removed

Late in experiment C3, two of the mice which had been given ovalbumin in water-in-oil emulsion showed sudden increases in titre as shown in table 50. These increases had the characteristics of a secondary response which may have been caused by the escape of emulsion from an encapsulated depot whilst the mice were being firmly held for bleeding.

An attempt was therefore made to produce this effect artificially using the four of the remaining eight mice which did not show high titres.

Each mouse was anaesthetized with ether. The adjuvant depot was located by palpation and the hair over the site clipped. A small slit was then made with a scalpel and the depot delivered through this and held with forceps.

As much of the content as possible was sucked into a syringe through a 1 mm needle. The depot was then released and the incision dusted with a wound dressing powder.

The volume of emulsion collected from each depot was insufficient for re-injection due to the dead volumes in syringe and needle. Some oil/Arlacel was therefore added to make up the volume to about 0.3 ml. This was then injected subcutaneously on the other side of the same mouse. The needles and syringe were flushed out several times with oil/Arlacel between each operation.

Serum samples were collected on the 4th, 8th and 204th days after these manipulations. The titres of these are given in table 51. The results show that there was a marked increase in titre following the treatment. In the case of mouse number 132, the depot burst on delivery through the incision and it was only possible to collect a very tiny amount of the contents for re-injection. There was no response in this mouse.

Table 52 gives the titres of the four remaining animals in the group which neither showed a natural secondary response, nor were re-injected.

Table 50. Antibody titres of mice given ovalbumin in water-in-oil emulsion in experiment C3a, which showed spontaneous secondary responses.

| Mouse number | Days from first inoculation | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 114 | 142 | 170 | 198 | 226 | 266 | 296 | 320 | 365 | 383 |
| 136 | 20 | 20 | 60 | 60 | 60 | 4860 | 4860 | 1620 | 1620 | 1620 |
| 137 | 60 | 60 | 60 | 180 | 180 | 1620 | 540 | 540 | 180 | 60 |

Table 51. Antibody titres of mice reinjected with the contents of their own water-in-oil emulsion depots.

| Mouse number | Days from first inoculation | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 198 | 226 | 266 | 296 | 320 | 365 | 379 | 383 | 387 | 538 |
| 130 | 1620 | 180 | 540 | 540 | 540 | 1620 | X | 540 | 4860 | 1280 |
| 132 | 180 | 60 | 180 | 180 | 180 | 540 | X | 540 | 540 | + |
| 135 | 20 | 20 | 20 | 60 | 20 | 180 | X | 540 | 4860 | 640 |
| 138 | 180 | 540 | 60 | 180 | 180 | 180 | X | 1620 | 4860 | 320 |

X = re-injection of depot. + = dead.

Table 52. Antibody titres of individual mice given ovalbumin in water-in-oil emulsion, experiment C3a.

| Mouse number | Days from first inoculation | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | 142 | 170 | 198 | 226 | 266 | 296 | 320 | 365 | 383 | 387 | 538 |
| 131 | 4,860 | 14,580 | 4,860 | 14,580 | 4860 | 4860 | 1620 | 4860 | 4860 | 4860 | 640 |
| 133 | 4,860 | 4,860 | 4,860 | 4,860 | 1620 | 4860 | 4860 | 1620 | 4860 | 4860 | 5120 |
| 134 | 10 | 10 | 10 | 10 | 10 | 10 | 5 | 10 | 10 | 10 | 10 |
| 139 | 14,580 | 43,740 | 14,580 | 14,580 | 4860 | 4860 | 4860 | 4860 | 4860 | 4860 | 2560 |

It will be seen from the tables that in this experiment three-fold, instead of doubling, dilutions were used for most of the serum titrations.

10. The inoculation of material from water-in-oil depots into other mice

The groups of mice given ovalbumin in a water-in-oil emulsion which had been further diluted with oil (experiment D3) gave an excellent antibody response. As a check on the extent to which antigen had survived in the depots of these mice, the depot contents were collected and inoculated into fresh mice.

The mice were destroyed with ether on the 271st day after the first injection. The skin was peeled back and as much material as possible collected from the depots of each group with a syringe. This material was then injected subcutaneously into fresh mice.

Serum samples were collected from the recipient mice on the 52nd day and titrated. The results are given in table 53. It is apparent that ample antigen remained in the depots to stimulate a good antibody response in the second set of mice.

Table 53. Titres of sera collected from mice inoculated with a water-in-oil emulsion, containing ovalbumin, recovered from the depots of other mice inoculated earlier.

| Type of inoculum given to original mice | Original dose of ovalbumin per mouse | Original number of mice (Grey) | Number of mice surviving | G.M.T. of group on the day that the depots were collected (27lst day) | Volume of emulsion available for injection into each new mouse | Number of new mouse (White) | Titre on the 52nd day |
|---|---|---|---|---|---|---|---|
| Neat emulsion | 2 mg | 5 | 3 | 254 | 0.05 ml | 500 | 1280 |
| | | | | | | 501 | 20 |
| | | | | | | G.M.T. = | 160 |
| Emulsion diluted 1:5 with oil/Arlacel | 0.4 mg | 10 | 7 | 2560 | 0.15 ml | 494 | 20 |
| | | | | | | 495 | 160 |
| | | | | | | 496 | 640 |
| | | | | | | G.M.T. = | 127 |
| Emulsion diluted 1:20 with oil/Arlacel | 0.1 mg | 10 | 9 | 493 | 0.15 ml | 497 | 5120 |
| | | | | | | 498 | 160 |
| | | | | | | 499 | 2560 |
| | | | | | | G.M.T. = | 1280 |

G.M.T. = geometric mean titre.

## 11. Investigation of the long term persistence of antigen in a mineral oil depot by transferring the emulsion to several mice in sequence

Immediately after chromatographically purified ovalbumin became available, three mice were each given 1 mg emulsified in Freund's complete adjuvant (Difco). The intention was to raise a high titred serum in them. Subsequently these mice were given two secondary injections of ovalbumin in normal saline as shown in table 54. The table also gives the geometric means of three serum samples collected from the mice.

On the 183rd day after inoculation they were killed with ether and a post-mortem carried out. The bodily condition of all three mice was normal and there was no marked reaction at the injection sites.

The depots were well encapsulated with fibrous tissue and it was quite easy to suck up the contents of each with a syringe. The volume of material obtained in this way was only 0.2 ml so another 0.2 ml of Incomplete Adjuvant (Difco) was mixed with it in the syringe. Two fresh mice were each inoculated subcutaneously with 0.2 ml of the mixture. Sera were collected at intervals over a year. The titres of these are given in table 55.

One of the two mice died on the 360th day and the other was killed with ether on the 361st day. The depot site in the latter was unremarkable and it was possible to collect some of the contents. Enough mineral oil (Drakeol 6-VR) was mixed with this to make the colume up to 0.4 ml and a further two mice were inoculated.

These, the third group of mice to which the emulsion had been passed, showed an antibody response (table 56) though it was much more feeble than that given by the first two groups.

The responses of all the three groups of mice in which this same emulsion was used are shown on the graph in figure 65.

Post-mortem examination of the injection site of one of the third pair of mice which survived to the 181st day showed that there was quite a lot of fibrous tissue reaction enclosing small oil drops. These drops were clear and appeared to have no creamy contents.

As a control, another group of four mice were inoculated with an emulsion prepared from Freund's Complete Adjuvant (Difco) but not containing ovalbumin. These mice were observed for 80 days and sera collected from them. None of these sera showed antibodies against ovalbumin when tested with sensitive batches of fresh tanned cells.

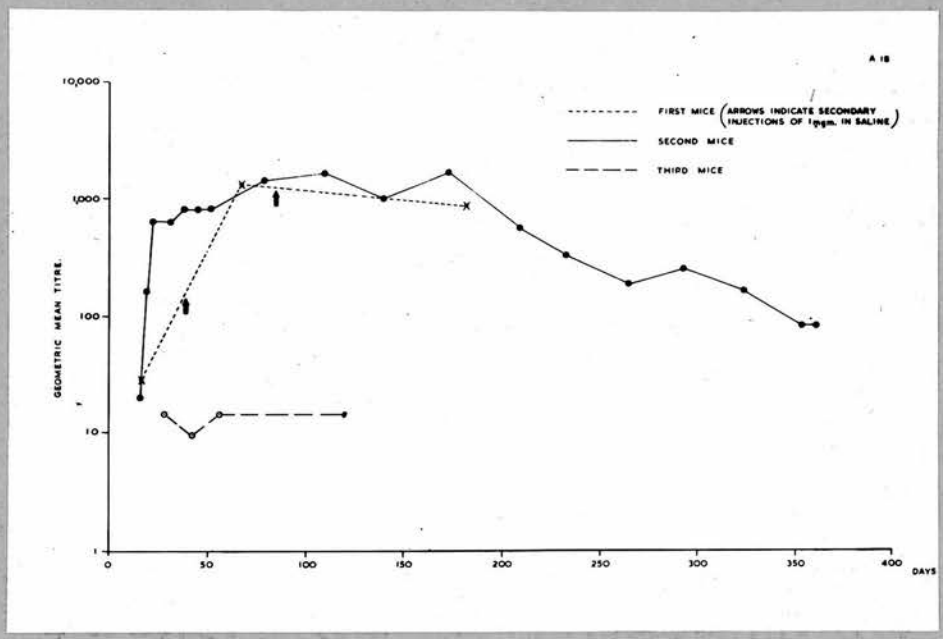



Fig.65. The antibody responses of three groups of mice to the same water-in-oil emulsion containing ovalbumin. The emulsion was recovered from each group of mice when they were killed and inoculated into fresh mice.

Table 54. Geometric mean titres of a group of three grey mice inoculated with ovalbumin in Freund's complete adjuvant.

| Days after first injection: | 0 | 16 | 44 | 68 | 85 | 183 |
|---|---|---|---|---|---|---|
| Dose of ovalbumin: | 1 mg | - | 1 mg | - | 1 mg | - |
| Vehicle: | Freund's complete | - | saline | - | saline | - |
| Titre: | 0 | 28 | ND | 1280 | ND | 806 |

ND = not done.

Table 55. Geometric mean titres of a second group of mice inoculated — with ovalbumin in Freund's complete adjuvant which had been recovered from a first group of mice on the 183rd day after injection.

| Number of mice (Grey) | Mean weight | Days after injection | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 16 | 19 | 22 | 31 | 38 | 45 | 52 | 79 | 110 | 140 | 173 | 201 | 233 | 265 | 293 | 324 | 354 | 361 |
| 2 | 31.0 g | 0 | 20 | 160 | 640 | 640 | 810 | 810 | 810 | 1403 | 1620 | 934 | 1620 | 540 | 312 | 180 | 240 | 160 | 80 | 80 |

Table 56. Geometric mean titres of the third group of mice inoculated with ovalbumin in Freund's complete adjuvant, which had been recovered from another group of mice 360 days after its inoculation into them.

| Number of mice (Grey) | Days after injection | | | |
|---|---|---|---|---|
| | 28 | 42 | 56 | 117 |
| 2 | 14.1 | 9.6 | 14.1 | 14.1 |

12. The response of mice to very small doses of antigen

It was just possible that the poor response of mice to ovalbumin in saline (experiment A1), in contrast to their response to the antigen in oil emulsion, was due to the paralysing effect of an overdose. If this was so then the superiority of the adjuvant emulsion might be due to the fact that it had locked up most of the antigen leaving only an optimum dose free to stimulate the antibody forming cells. In the preliminary experiments 500 µg was the lowest dose used. The ability of very tiny doses of ovalbumin to raise antibodies in mice was therefore tested.

The mice in seven groups of five were each inoculated with a single dose of from 0.25 to 1280 µg of ovalbumin in saline. Sera were collected on the 12th, 16th and 20th days and titrated with a very sensitive batch of fresh tanned cells. The results are given in table 57 and are plotted as a dose response curve in figure 66. They give no evidence that an overdose was being used.

On the 30th day after inoculation all the mice were given a secondary injection of 0.5 mg ovalbumin each in saline. A final serum sample

was collected nine days later and titrated. These titrations were done with preserved cells 64 fold less sensitive than the fresh ones used previously. Negative results may not therefore be fully indicative of there having been no sensitization by the primary injection.

Once again, these results show that at least 1.0 mg of ovalbumin in saline must be given to obtain a useful response. The standard dose of 2 mg of ovalbumin per mouse was not excessive.

Table 57. Geometric mean antibody titres of groups of mice inoculated with ovalbumin in saline.

| Dose | Number of mice (Grey) | Mean weight | Days after first inoculation | | | | |
|---|---|---|---|---|---|---|---|
| | | | 12 | 16 | 20 | 30 | 39 |
| 0.25 µg | 5 | 30.7 g | 0 | 0 | 0 | S | 0 |
| 1.0 µg | 5 | 30.4 g | 0 | 0.02 | 0 | S | 0 |
| 4.0 µg | 5 | 30.7 g | 0.02 | 0.03 | 0 | S | 3.7 |
| 16 µg | 5 | 30.5 g | 0.05 | 0.04 | 0.03 | S | 0 |
| 64 µg | 5 | 30.1 g | 0.02 | 0.03 | 0.02 | S | 0 |
| 256 µg | 5 | 30.7 g | 0.08 | 0.40 | 0.38 | S | 26.7 |
| 1280 µg | 5 | 30.7 g | 0.11 | 0.25 | 0.56 | S | 242 |

0 = no antibodies detected.

S = secondary inoculation of 0.5 mg of ovalbumin in saline.

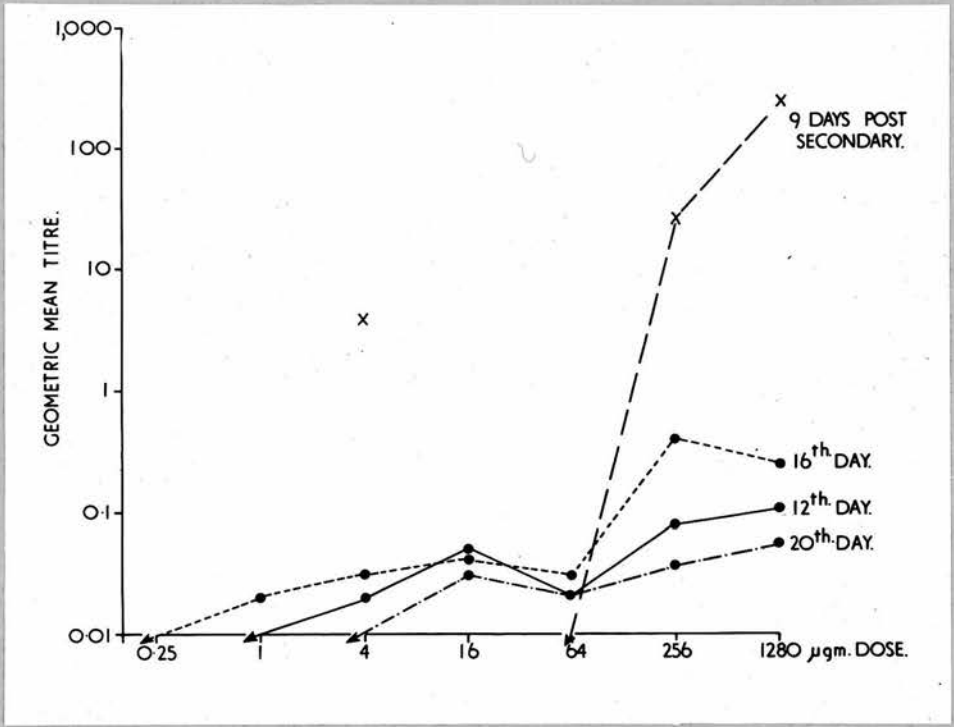



Fig.66. Dose response curves for groups of mice inoculated with doses of ovalbumin in saline from 0.25 to 1280 µg. A secondary injection of 0.5 mg of ovalbumin in saline was given to all groups on the 30th day.

## 13. Simulation of the slow release of antigen from a depot

Experiments D10 and D11 showed that even after a year, water-in-oil depots still contained a considerable amount of antigen. Experiment A4 showed that even as little as 2 µg of ovalbumin was effective in a water-in-oil emulsion. Thus only tiny quantities of antigen appeared to be necessary to maintain the high adjuvant response.

An attempt was therefore made to mimic the slow release from a depot by repeated injections of antigen, but without giving a high total weight of antigen.

It was assumed that there was a large release of antigen from the depot immediately after injection but that this was followed by a rapid drop to a minimum release rate. Ten mice were therefore each given a first dose of 64 µg of ovalbumin in saline followed by daily doses, reduced by a half each time. When 1 µg per mouse had been reached the daily dose was kept at this level for the rest of the experiment. Inoculations were stopped on the 50th day.

Table 58 gives the inoculation schedule and table 59 the results of serum titrations carried out at intervals. This latter table also shows the geometric mean titres of a typical group of

mice given 2 mg of ovalbumin each in water-in-oil emulsion. This was the control group of experiment B1.

The results in table 59 are shown as a graph in figure 67 where it can be seen that the curves for each group are quite close together until the 50th day. There is then a precipitous fall in the mean titre of those given the daily injections which were stopped on that day. These results show that the adjuvant response can be mimicked by daily injections of antigen in saline.

Table 58. Schedule of inoculations of ovalbumin in saline given daily to mice in an attempt to mimic the adjuvant response

| Day: | 0 | 1 | 2 | 3 | 4 | 5 | 6 to 50 | |
|---|---|---|---|---|---|---|---|---|
| Dose in µg: | 64 | 32 | 16 | 8 | 4 | 2 | 1 | daily | Total weight of ovalbumin injected = 171 µg |

14. A study of the type of antibody produced by mice during their response to antigen in water-in-oil emulsion

The object of this experiment was to find out to what extent IgM type antibody was being produced during the adjuvant type of sustained

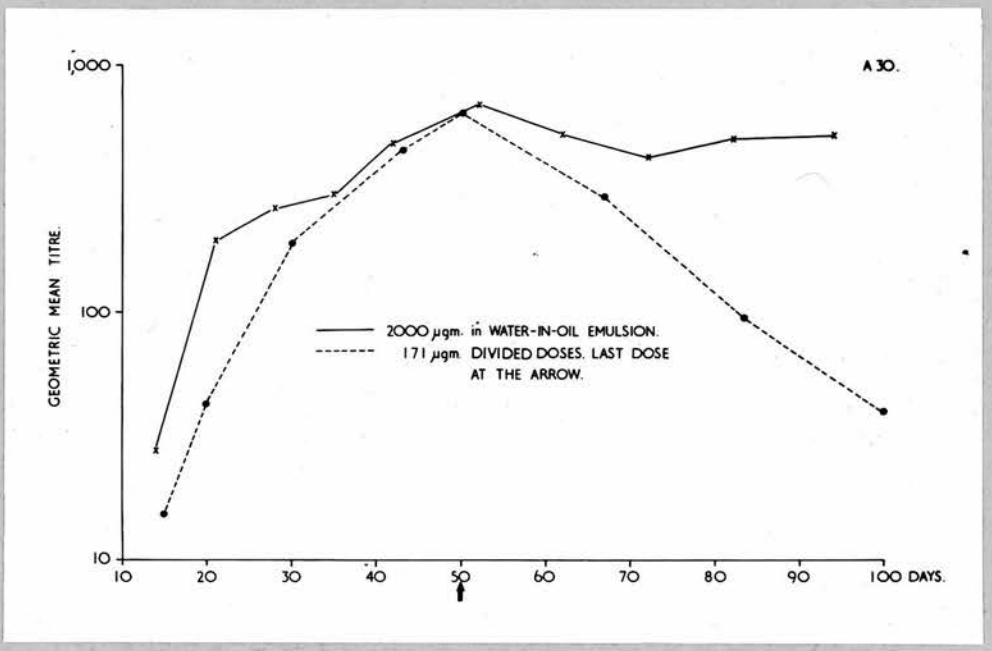



Fig.67. The antibody response curve of a group of mice inoculated with daily doses of ovalbumin in saline up to the 50th day, compared with the response of a group of mice to a single subcutaneous inoculation of 2 mg of ovalbumin in a water-in-oil emulsion.

292.

Table 59. Geometric mean titres of a group of mice given daily inoculations of ovalbumin in saline compared with those of a group of mice given a single 2 mg dose of ovalbumin in water-in-oil emulsion.

| Group | Number of mice (Grey) | Mean weight | Days after first injection | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 14 | 15 | 20 | 21 | 28 | 30 | 35 | 42 | 43 | 50 | 52 | 62 | 67 | 72 | 82 | 84 | 94 | 100 |
| Daily doses | 10 | 29.7 g | - | 15.1 | 42.6 | - | - | 190 | - | - | 452 | 640 | - | - | 293 | - | - | 95 | - | 40 |
| Single dose in water-in-oil emulsion (Expt B1) (Table 31) | 10 | 24.2 g | 27.6 | - | - | 196 | 260 | - | 296 | 484 | - | - | 689 | 520 | - | 422 | 508 | - | 520 | - |

response to an antigen in water-in-oil emulsion. If IgM in addition to IgG type antibody could have been demonstrated late in the response this might have been taken to indicate that a continual release of antigen was taking place (Bauer, Mathies and Stavitsky, 1963).

Two methods were used: a) the destruction of IgM antibodies with 2-mercaptoethanol, and b) the separation of IgM from IgG antibodies by gel filtration using Sephadex G 200.

a) Treatment with 2-mercaptoethanol

Sera from groups of mice responding to an inoculation of 2 mg of ovalbumin in water-in-oil emulsion were collected in pools on the 14th, 21st, 80th, 101st, and 178th days. Two other pools of sera were from similar mice, but these had been given secondary inoculations on the 20th day and the sera had been collected on the 4th, and 8th days after that.

All the sera were treated with 2-mercapto-ethanol at the same time and in the same way as described in the Methods section of this thesis. A control containing saline in place of the mercaptoethanol was set up for each serum.

After absorption with sheep cells to remove any non-specific agglutinins, the treated and

control sera were titrated with a sensitive batch of fresh tanned cells. The titrations were arranged in a random manner on the plates and were scored without reference to the key.

The results are shown in table 60. Only in the case of the serum taken on the 14th day was there any reduction in titre after treatment with 2-mercaptoethanol. The other titres show close agreement between those treated and the controls.

b) Gel filtration with Sephadex G 200

Three sets of sera were selected from those of mice responding to ovalbumin in water-in-oil emulsion.

The first was from mice on the 12th day after injection of 2 mg of ovalbumin in a water-in-oil emulsion. The pooled volume available was 0.8 ml.

The second set was from similar mice, but the sera had been collected on the 27th day. The pooled volume available was 1.0 ml.

The third set was from similar mice again, but in this case they had been given a secondary injection of 1 mg of ovalbumin in saline on the 40th day. The sera were collected on the fourth day after this. The pooled volume available was 0.8 ml.

Table 60. Results of the titration of sera from mice inoculated with ovalbumin in water-in-oil emulsion. Sera treated with 2-mercaptoethanol.

| Serum identification | Day on which collected | | Titres of treated and control sera | |
|---|---|---|---|---|
| | Post primary inoculation | Post secondary inoculation | | |
| A | 14 | - | Treated | 192 |
| | | | Control | 384 |
| B | 21 | - | Treated | 6,144 |
| | | | Control | 6,144 |
| C | 80 | - | Treated | >98,304 |
| | | | Control | >98,304 |
| D | 101 | - | Treated | >98,304 |
| | | | Control | >98,304 |
| E | 178 | - | Treated | 98,304 |
| | | | Control | 98,304 |
| F | 24 | 4 | Treated | 12,288 |
| | | | Control | 12,288 |
| G | 28 | 8 | Treated | 49,152 |
| | | | Control | 49,152 |

Each pool was passed through a gel filtration column of Sephadex G200 as described in detail in the Methods section. The smaller protein molecules in serum enter this gel-like material and their passage through the column is delayed. The large molecules including the IgM antibodies cannot enter the gel structure, so they are swept on by the buffer and appear first in the eluate.

For the pools of sera numbered <u>one</u> and <u>three</u>, a column as described under Methods was used. This was prepared with about 20 g (dry weight) of Sephadex G200. The height of the column was 67 cm corresponding to a volume of about 3370 ml. For the sera reported as the <u>second</u> pool, the column was re-made, using 28 g of Sephadex. This gave a height of 90 cm and a volume of about 4520 ml.

The protein content of each fraction (4.0 or 7.8 ml) was measured with the ultraviolet spectrophotometer and recorded as optical density at 280 mμ. The graphs, figures 68, 69, and 70, show the curves drawn from these measurements. In each case the first peak contains IgM antibodies, the second peak contains IgG antibodies and the third peak small molecules such as serum albumin (Flodin and Killander, 1962). It is

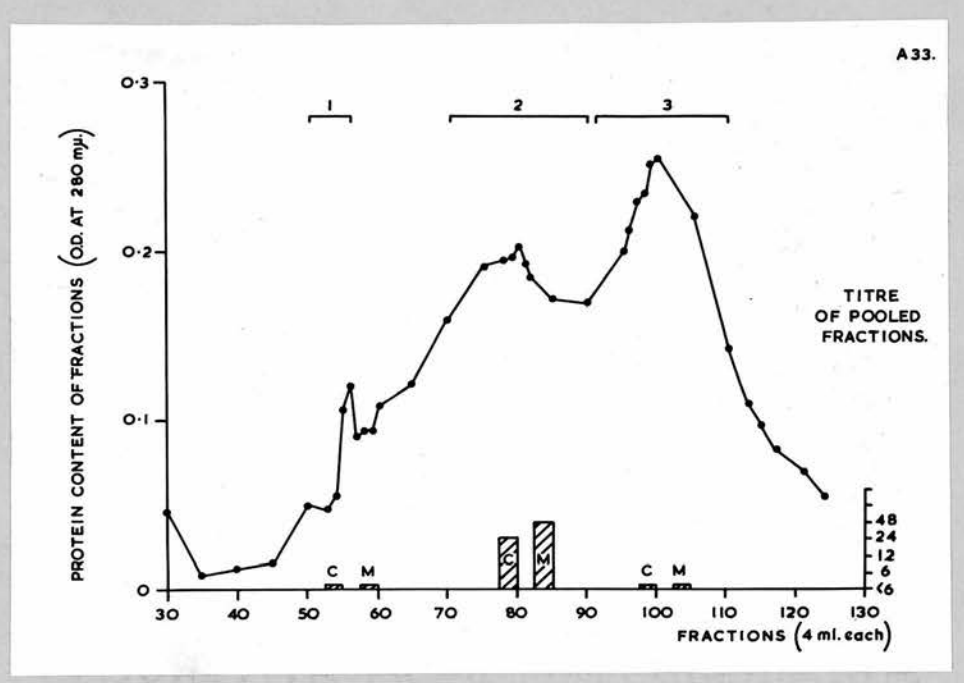



Fig.68. Separation of serum proteins on a Sephadex G200 column. Serum collected from mice 12 days after an inoculation of 2 mg of ovalbumin in a water-in-oil emulsion.

Continuous line = protein content of the fractions.
The square brackets indicate the fractions pooled for titration.
Bars at the bottom of the graph represent tanned cell titres of these pools. C = control.
M = treated with 2-mercaptoethanol.

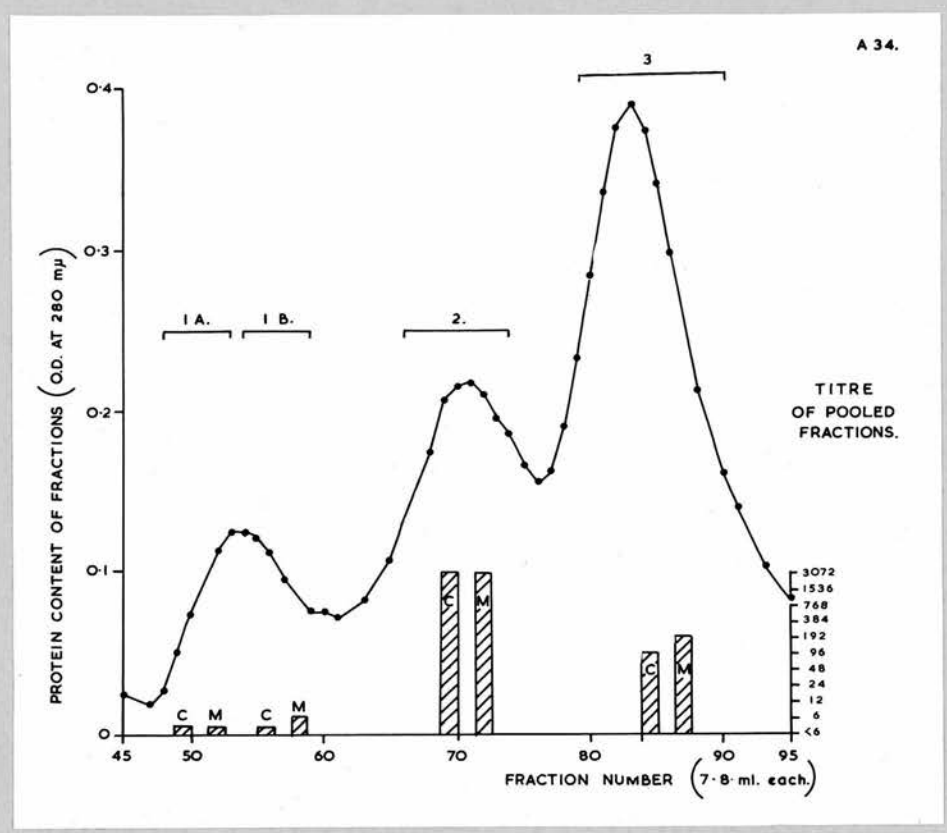



Fig.69. Separation of serum proteins on a Sephadex G200 column. Serum collected from mice 27 days after inoculation of 2 mg of ovalbumin in a water-in-oil emulsion.

Continuous line = protein content of fractions.
Square brackets indicate the fractions pooled for titration.
Bars at the bottom of the graph represent the tanned cell titres of these pools. C = control.
M = treated with 2-mercaptoethanol.

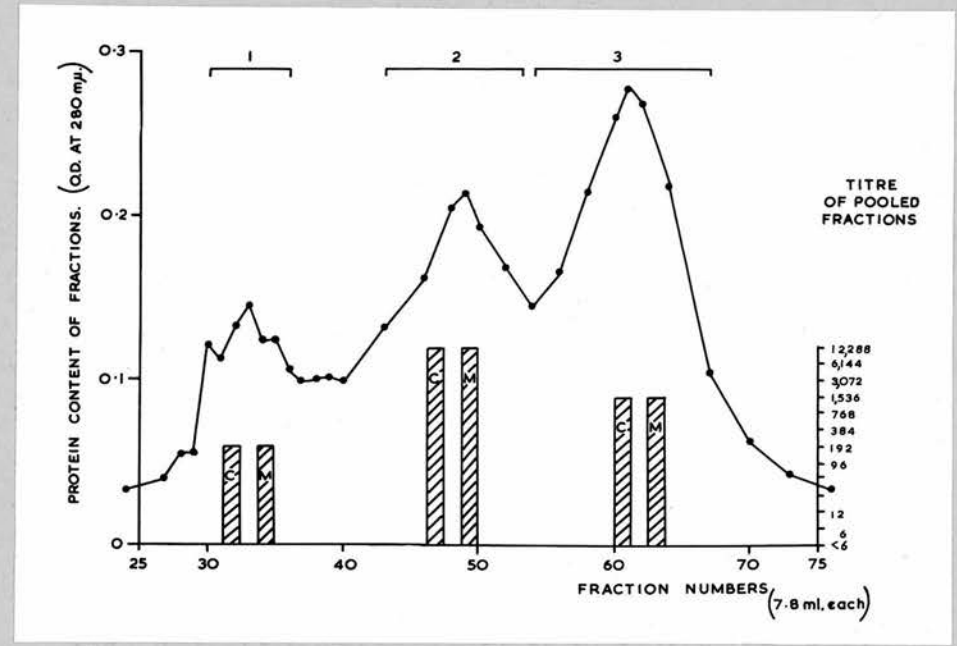



Fig.70. Separation of serum proteins on a Sephadex G200 column. Serum collected from mice 44 days after an inoculation of 2 mg of ovalbumin in a water-in-oil emulsion, and 4 days after a secondary stimulation with 1 mg of ovalbumin in saline.

Continuous line = protein content of the fractions.
Square brackets indicate the fractions pooled for titration.
Bars at the bottom of the graph represent the tanned cell titres of these pools. C = control.
M = treated with 2-mercaptoethanol.

seen from these graphs that the separation of the peaks is not very sharp.

Certain fractions as indicated on the graphs by the square brackets, were pooled and concentrated down to the original serum volume by means of polyethylene glycol. These fractions were then treated with 2-mercaptoethanol exactly as described in section a) above. Controls were also set up. The results of the subsequent titrations with fresh tanned cells are given in table 61 and are also shown as bars on each graph.

No antibodies were detected which could be destroyed by 2-mercaptoethanol.

15. The antigenicity of small volumes of water-in-oil emulsion containing ovalbumin injected directly into lymph nodes

Freund and Lipton (1955) reported that particles of an injected emulsion reached regional lymph nodes within an hour of inoculation. They remained there and were antigenically effective even if the main depot was removed. As a check on the antigenicity of tiny depots of this type, very small quantities of water-in-oil emulsion containing antigen were injected directly into a lymph node.

Table 61. Results of the titration of fractions of sera from mice responding to ovalbumin in water-in-oil emulsion. Sera fractionated on Sephadex G200 and treated with 2-mercaptoethanol.

| Serum examined | Tanned cell titres | | | | | |
|---|---|---|---|---|---|---|
| | First peak (IgM) | | Second peak (IgG) | | Third peak | |
| | Control | M.C.E. treated | Control | M.C.E. treated | Control | M.C.E. treated |
| First serum (12th day after ovalbumin in adjuvant) | < 12 | < 6 | 24 | 48 | < 12 | < 6 |
| Second serum (27th day after ovalbumin in adjuvant) | a) < 6 | < 6 | 3,072 | 3,072 | 96 | 192 |
| | b) < 6 | 6 | | | | |
| Third serum (44th day after ovalbumin in adjuvant; 4th day after secondary) | 192 | 192 | 12,288 | 12,288 | 1,536 | 1,536 |

A water-in-oil emulsion containing 2 mg of ovalbumin in each 0.2 ml was prepared by the syringe method. Five mice were each anaesthetized with ether, their inguinal lymph nodes (Dunn, 1953) exposed and an injection of between 0.5 and 1.0 µl of emulsion made directly into the substance of the node through a fine glass tube. This volume was equivalent to a dose of ovalbumin of between 5 and 10 µg. In most cases there was no leakage of emulsion and it could sometimes be seen within the node before this was replaced. The wounds were dressed and all healed without complication. Further details of the technique employed are given in the Methods section.

The inguinal lymph nodes were chosen as ovalbumin, fluorescently labelled by the method of Nairn (1962) and injected subcutaneously over the back, was seen to drain to these nodes.

Sera were collected at intervals and titrated. The results are given in table 62. They are also shown in the graph figure 71 together with the curves for 2 µg and 20 µg of ovalbumin in water-in-oil emulsion from experiment A4 (table 30). The curve for the present experiment lies between them. A depot within a lymph node therefore appears to be no more nor no

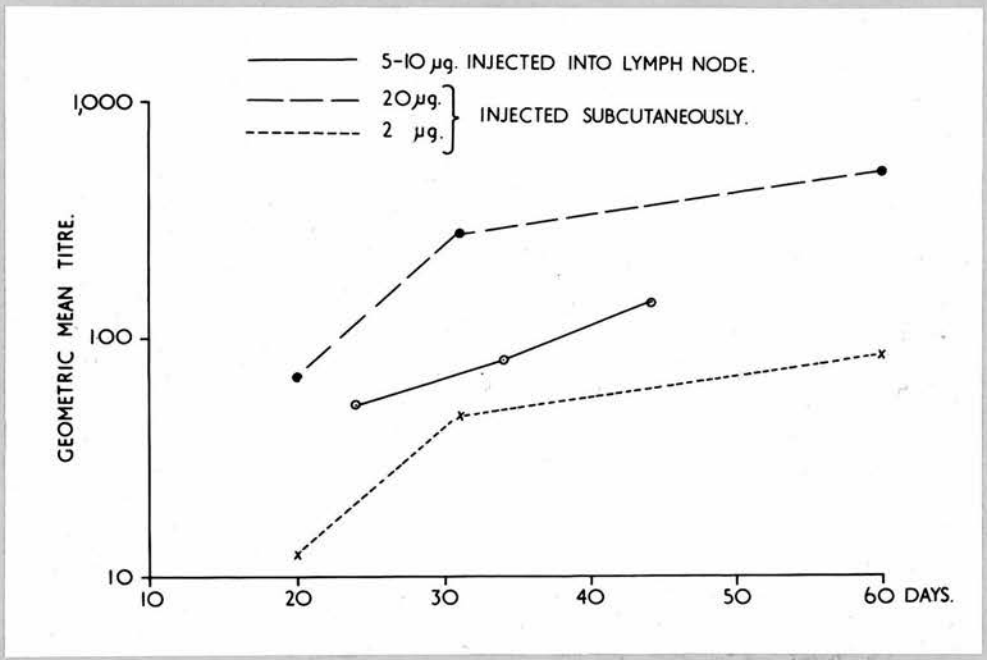



Fig.71. The antibody response of mice to the injection of a small quantity of water-in-oil emulsion (containing ovalbumin) into an inguinal lymph node. This is shown by the continuous line.

Broken lines show the antibody responses of mice given 2 and 20 µg of ovalbumin in 0.2 ml of water-in-oil emulsion, subcutaneously.

less effective than one placed subcutaneously.

When a post-mortem examination of these mice was carried out on the 55th day, no emulsion was detected in the injected nodes. They were examined with a hand lens only.

Table 62. Geometric mean titres of mice given an injection of 5-10 µg of ovalbumin in a small volume of water-in-oil emulsion directly into a lymph node.

| Number of mice | Days after the injection | | |
|---|---|---|---|
| | 24 | 34 | 44 |
| 5 | 52 | 80 | 140 |

16. A comparison of the *in vivo* and *in vitro* break-down of water-in-oil emulsions

As has been reported in the results of experiments B2 and D3, post-mortem examination of water-in-oil emulsion depots which had remained in mice for a long period showed that they had a stratified appearance as shown in figure 72 (left).

It was observed that water-in-oil emulsions left undisturbed on the bench for some months after preparation showed a similar stratification. As they broke down, clear oil rose to the top and a layer of water, containing antigen, appeared at the bottom. This is illustrated in figure 72

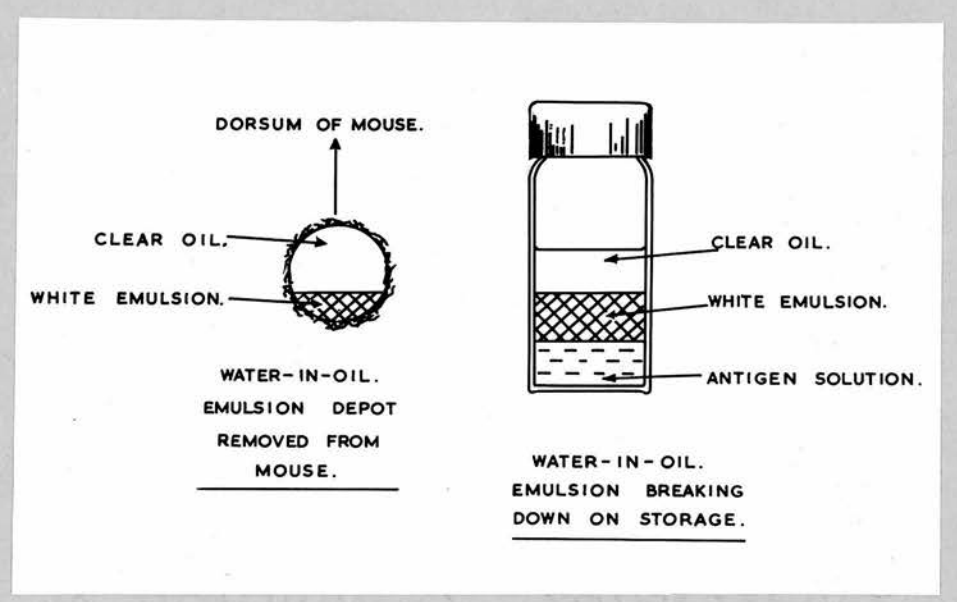



Fig. 72. Left. Water-in-oil emulsion depot removed from a mouse 6 - 8 months after inoculation. Right. Water-in-oil emulsion after some months storage at room temperature.

(right). That antigen was present in the lowest layer was confirmed by observing the break-down of a water-in-oil emulsion containing ovalbumin labelled with fluorescein by the method of Nairn (1962). This ovalbumin was seen in the bottom layer. A similar stratification was seen in the water-in-oil emulsions in Oakley-Fulthorpe immunodiffusion tubes as reported in experiment D5 (table 47).

It was found that freshly prepared water-in-oil emulsions of the standard type could be centrifuged at 750 G for 10 minutes without breaking. There was no separation of a water phase, but a layer of clear oil appeared at the top. This oil was equivalent to about 10 per cent of the total volume. The relative volume of oil seen in stratified depots is much greater than this, suggesting that emulsion break-down has occurred with slow release of antigen from the bottom of the depot.

E. A comparison, in man, of saline and water-in-oil emulsion influenza virus vaccines

This work was carried out in collaboration with Dr. J.R. Philp and Dr. S. Selwyn, who collected the serum samples and carried out the inoculations, and with the assistance of other members of the Bacteriology Department, University of Edinburgh Medical School.

The object of the study was to examine the local reactions and antibody response of man to a water-in-oil emulsion influenza virus vaccine which it was proposed to market in this country. The tests were controlled with a saline vaccine.

Both vaccines were prepared by Evans Medical Ltd. The saline vaccine 'Munevan' contained 7,500 haemagglutinating (HA) units of formalin killed virus antigen A2/Singapore/1/57, 2,500 HA units of A2/England/1/61, and 5,000 HA units of B/England/939/59, in each dose of 1 ml. The water-in-oil adjuvant vaccine 'Admune' contained one fifth the quantity of each of these antigens in each 0.25 ml dose.

The adjuvant vaccine had been prepared by the method of Himmelweit (1960) using the mineral oil Drakeol 6-VR and the emulsifier Arlacel A.

The saline vaccine was given by subcutaneous inoculation, and the adjuvant vaccine by deep

intramuscular injection to medical students and volunteers from an industrial laboratory. Forty-two students and 32 laboratory staff received the adjuvant vaccine and 46 students and 34 laboratory staff received saline vaccine. The students were aged between 20 and 26 years and the laboratory staff between 21 and 59 years, the mean age of the latter being 31 years. The age distributions of the groups are set out in table 63.

Blood samples were collected at inoculation and 1, 4, and 12 months later from the students; and at inoculation and 1 and 8 months later from the laboratory staff. The sera were removed from these and stored at $-30^{\circ}$. They were titrated in pairs or triplets (of sera from each volunteer) by the standard plastic plate method (W.H.O. 1953) using four haemagglutinating units of the antigens A2/Singapore/1/57 and B/England/939/59. Both absolute titres and fold changes in titre between pairs were recorded.

Table 64 gives the geometric means of these results for each group. Table 65 gives the fold changes in titre shown by members of the student group whose initial pre-vaccination titres were higher or lower than the mean. Those with the low initial titres gave the best response.

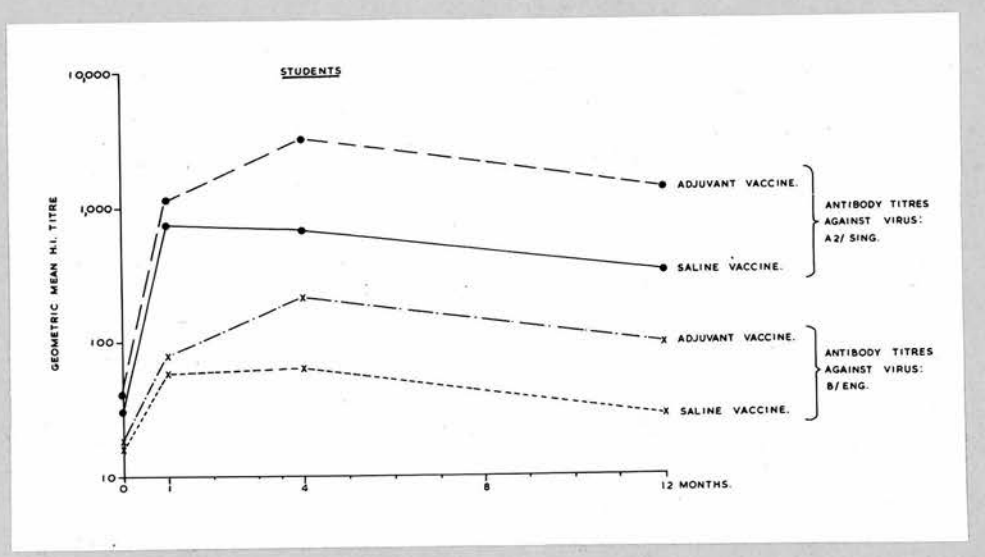
There was no difference between the two vaccine groups.

Table 66 gives the titres of antibody against the B strain virus shown by different age groups of the laboratory staff eight months after vaccination. There was no evidence that one age group was responding more strongly than the others to the B strain virus in the vaccine.

The graphs in figure 73 show the overall changes in titre for both groups. Figure 74 gives histograms of the fold changes in titre in the student group between the pre-immunization and three subsequent bleeds.

Reactions to the injections were recorded from the students by individual questioning one week after the inoculations had been given. Those for the industrial laboratory staff were recorded whilst the second blood sample was being taken at one month. The reactions reported are given in table 67. None of the volunteers developed any severe general or local reactions.

The 'general reactions' column in the table refers to volunteers reporting malaise, slight fever, etc., which occurred soon after the injection. Pain at the injection site tended to persist for a longer period in those given adjuvant than in those given saline vaccine.



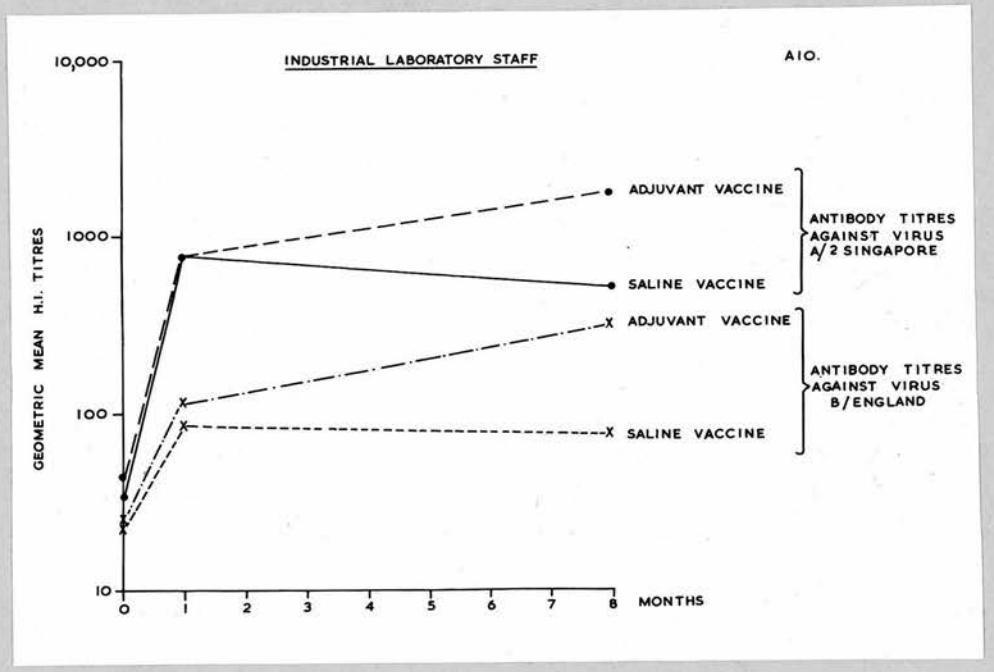




Fig.73. Geometric mean haemagglutination inhibiting antibody titres against A2 and B strains of influenza virus.

Above: Students, Below: Industrial laboratory staff inoculated with influenza virus vaccine (mixed strains) in either a saline or oil adjuvant vehicle.

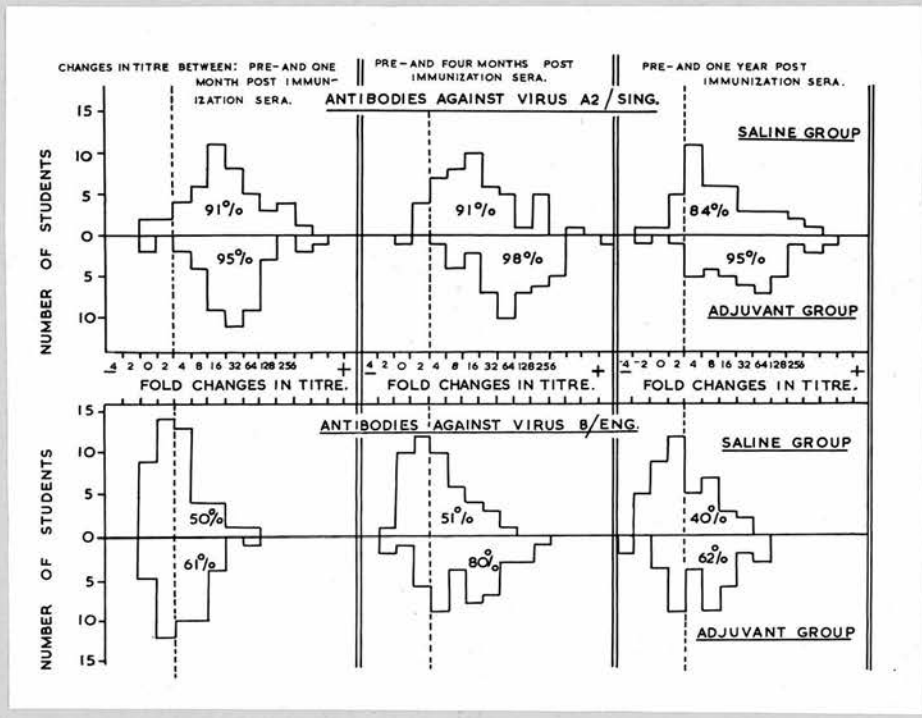



Fig.74. The numbers of students showing the indicated fold changes between pre- and 1, 4, or 12 month post inoculation H.I. antibody titres. The students were divided into two groups inoculated with either a saline or oil adjuvant, mixed strain, influenza virus vaccine.

This was probably associated with the fact of an intramuscular injection having been given. The pain was in no case severe. The minor lesions were areas of erythema and swelling persisting for several days. It is notable that these were much more frequent in the laboratory staff group. In most of these the lesion was not noticed by the volunteer himself but was pointed out to him by his wife.

Table 63. Age distribution of students and industrial laboratory staff inoculated with saline or oil adjuvant influenza virus vaccine.

| Group | Age in years | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 | 28 | 29 | 30 | 31-35 | 36-40 | 41-45 | 46-50 | 51-55 | 56-60 |
| Industrial laboratory staff | - | 1 | 3 | 4 | 2 | 9 | 6 | 3 | 4 | 2 | 2 | 15 | 12 | 1 | 2 | - | 2 |
| | | | | 19 | | | | | 17 | | | | | | | | |
| Students | 10 | 35 | 25 | 12 | 3 | 3 | 1 | - | - | - | - | - | - | - | - | - | - |

Table 64. Geometric mean antibody titres before and after administration of saline and adjuvant influenza vaccines.

| Vaccination groups and antigen | Before | 1 month after | 4 months after | 8 months after | 12 months after |
|---|---|---|---|---|---|
| Students | | | | | |
| Saline A2 | 31 | 747 | 639 | - | 322 |
| Adjuvant A2 | 41 | 1,166 | 3,080 | - | 1,324 |
| Saline B | 16 | 58 | 64 | - | 28 |
| Adjuvant B | 18 | 78 | 210 | - | 94 |
| Laboratory staff | | | | | |
| Saline A2 | 34 | 785 | - | 507 | - |
| Adjuvant A2 | 38 | 784 | - | 1,743 | - |
| Saline B | 22 | 87 | - | 76 | - |
| Adjuvant B | 25 | 116 | - | 312 | - |

Final serum specimens were obtained from 43 students and 26 laboratory staff receiving saline vaccine, and from 40 students and 26 laboratory staff receiving adjuvant vaccine.

Table 65. Fold increases in titre shown by students, with high or low initial antibody titres, by the fourth month after inoculation.

| Vaccine | Virus | Pre-vaccination titre | Number of students | Mean fold changes in titre between initial and fourth month serum sample | $Log_2$ fold change | $Log_2$ difference between the groups |
|---|---|---|---|---|---|---|
| Saline | A2 | 1:80 or higher | 14 | 9.2 | 3.1 | 2.5 |
| | | 1:10 or below | 7 | 48.0 | 5.6 | |
| | B | 1:40 or higher | 15 | 2.3 | 1.1 | 2.7 |
| | | 1:5 or below | 8 | 13.9 | 3.8 | |
| Adjuvant | A2 | 1:80 or higher | 14 | 26.0 | 4.7 | 3.6 |
| | | 1:10 or below | 7 | 313 | 8.3 | |
| | B | 1:40 or higher | 12 | 7.4 | 2.9 | 1.8 |
| | | 1:5 or below | 3 | 26.0 | 4.7 | |

Table 66. Geometric mean titres of antibody against influenza virus B shown by different age groups of the industrial laboratory staff.

| Vaccine | Age-group (years) | Pre-vaccination | | One month | | Eight months | |
|---|---|---|---|---|---|---|---|
| | | Number of staff | Mean titre | Number of staff | Mean titre | Number of staff | Mean titre |
| Saline | 21 - 25 | 11 | 26 | 11 | 91 | 8 | 95 |
| | 26 - 30 | 8 | 22 | 8 | 67 | 7 | 72 |
| | 31 - 59 | 15 | 20 | 15 | 96 | 11 | 66 |
| Adjuvant | 21 - 25 | 8 | 16 | 8 | 56 | 7 | 195 |
| | 26 - 30 | 7 | 20 | 7 | 176 | 4 | 453 |
| | 31 - 59 | 17 | 32 | 17 | 142 | 15 | 351 |

Table 67. Reactions to saline and adjuvant influenza vaccines.

| Vaccination groups | No. | General reactions | Local reactions | | | | Total with reactions |
|---|---|---|---|---|---|---|---|
| | | | Pain (duration in days) | | | Minor lesions | |
| | | | 1 | 2-3 | 4-5 | | |
| Students/saline | 46 | 4 | 24 | 7 | 0 | 5 | 35 |
| " /adjuvant | 42 | 7 | 9 | 18 | 4 | 0 | 33 |
| Laboratory staff/saline | 34 | 3 | 6 | 9 | 2 | 17 | 20 |
| " /adjuvant | 32 | 0 | 3 | 12 | 1 | 1 | 17 |

# Part Six

# DISCUSSION

## DISCUSSION

In the following discussions, the information which has been obtained relating to the mode of action of the water-in-oil emulsion adjuvants will be examined first. The practical aspects of the use of this type of adjuvant will then be discussed with particular reference to the influenza virus vaccine trial which was carried out as part of the study.

The results of the special investigations of certain of the materials and methods used: ovalbumin and the tanned red cell agglutination test, have been discussed earlier on pages 136 and 193.

### A. The mode of action of the mineral-oil emulsion adjuvants

The many theories developed to account for the action of the depot-forming adjuvants have been presented in the Introduction to this study. The chief of these suggest that:

a. The adjuvant material has itself some stimulating action on the antibody forming apparatus.

b. A special antibody-forming 'organelle' is formed round the depot.

c. Antigen is held in the depot and released

slowly so producing a continuous series of secondary responses.

d. The antigen is presented to the antibody-forming apparatus in a more acceptable form.

Many investigators believe that a combination of these effects is needed to account for the extraordinary efficiency of this type of adjuvant in stimulating antibody production. The most common view of the practical vaccine maker is summed up in the words of Davenport (1961, page 178): "...Thus the emulsified "inoculum appears to serve not only as a local "protected depot of antigen, but as a source of "emulsified antigen which slowly and progressively "leaves the depot in a shielded state and "reaches, through the lymphatic system, remote "foci, where, it is believed, the emulsion breaks "down and antibody production is stimulated in "suitable cells. Probably the macrophages which "accumulate about the antigen depot play an "important role in the transportation of the "emulsion droplets.... The suggestion has also "been made that the mineral oil component of the "emulsion causes a hypertrophy and hyperplasia of "the cells capable of forming antibody and there-"by prepares them to function more effectively "upon the arrival of antigen."

The present results suggest that though this view may be broadly correct, in fact it gives a sufficiently false impression of the situation to hamper the rational use of this type of adjuvant. In this discussion it will be suggested that the only function of the mineral oil emulsion is to form a long term protected depot of antigen. Tiny quantities of antigen escape from the emulsion as it slowly breaks down. This antigen is sufficient to stimulate the antibody forming apparatus to produce a high level of antibody over a long period. Oil or emulsion does not have to leave the depot area and the fibrous encapsulation which develops is insufficient to prevent the escape of antigen into the circulation. There is no evidence that any sort of organelle is formed nor that any part of the adjuvant has a specific stimulating effect on the antibody producing cells.

In short, none of the experiments carried out gave any reason to doubt that the 'Depot Theory of Glenny' (Glenny, Buttle and Stevens, 1931) is correct and fully able to account for the results obtained.

Factors which were found not to be responsible for the adjuvant effect

The results of a number of experiments

showed that some apparently promising factors could be discarded as being responsible for the adjuvant action of the water-in-oil emulsions.

Materials such as lanolin, which could be used as emulsifiers, have been shown to stimulate antibody production to an antigen mixed with them in the absence of oil (Ramon, Richou and Staub, 1937) Dale (1961) found that both Arlacel A and the mineral oil Bayol F as well as complete water-in-oil emulsion caused lymphatic proliferation at the injection site. This she believed might be related to their adjuvant effect. She did not test the separate materials for their ability to stimulate antibody production.

Examination of the adjuvant effect of complete water-in-oil emulsions by injecting them at a different site to the antigen have usually been carried out with water-in-oil adjuvant containing Mycobacteria. This does cause an enhanced response (Bishop, 1961; Muramatsu, 1964), presumably due to the known stimulating effect of the mycobacterial waxes (Pound, 1958; White et al., 1958). Halbert et al. (1946) reported that when a plain water-in-oil emulsion was injected apart from the antigen there was no enhancement of antibody formation and Bernstein and Malkiel (1964) found no alterations in

existing viral, bacterial or pollen antibody titres after injections of a saline in water-in-oil emulsion placebo in man.

The results obtained from the present experiments gave no reason to suppose that the oil or the emulsifier or the complete emulsion produced their adjuvant effect by a direct stimulation of the reticulo-endothelial system or of any part of the haemopoietic apparatus.

(i) There was no significant difference between the response of mice inoculated with ovalbumin alone or in the presence of the emulsifiers Arlacel A or Tween 80 (table 43 and fig. 58). This does not, however, entirely rule out the possibility that Arlacel A, when held in the oily depot, may exert some effect on the lymphatic tissues causing better utilization of the antigen released. An adjuvant type of response was seen after the inoculation of ovalbumin incorporated into an oil/Arlacel mixture without a water phase, but not after the inoculation of ovalbumin incorporated into the oil alone (table 46 and fig. 60). However, a physical explanation of this effect is more probable (see below) than that the Arlacel had some stimulant action.

(ii) When a water-in-oil emulsion, containing another antigen, was inoculated 18 or

81 days after a first injection of ovalbumin in water-in-oil emulsion no effect was noted on the response curve to the first antigen (figs. 49 and 50). If there had been some direct stimulating effect of the adjuvant materials on the antibody forming cells, some change in the shape of the response curve to the first antigen would have been expected. It remained a flat plateau closely following the response shown by the controls which had not been given a second injection (fig. 51).

(iii) A water-in-oil emulsion injected before, or after, or at the same time as the antigen had no significant enhancing effect on antibody production (table 44). When the antigen was mixed in the same syringe with the emulsion there <u>was</u> an adjuvant effect (table 44, group D). A less marked and shorter lived increase in titre was also seen when ovalbumin was injected together with an oil-in-water emulsion (figs. 43 and 57).

These two results could be taken to indicate that there was some direct stimulation from the oil itself. The emulsifiers, as noted above, did not seem to have any effect (fig. 58). Other explanations could be that:

a) Due to the presence of emulsifiers, there was some automatic incorporation of antigen into the emulsion. Experiment D5 (page 267) showed that when oil/Arlacel is placed in the wells of an agar plate, or over the agar layer in a precipitation tube, tiny particles, probably of saline, become included in the mixture and appear as smoky swirls within it. These later become a layer at the bottom (table 47). The same effect is also seen <u>in vivo</u>: plain oil/Arlacel mixture injected subcutaneously develops a creamy stratum after some months (experiment D4, page 260).

When ovalbumin and the water-in-oil emulsion were mixed in the syringe, some ovalbumin may have entered the emulsion in this way. Only a little antigen would need to be incorporated: 20 µg of ovalbumin in a water-in-oil emulsion would be sufficient to produce an equivalent level of antibody (see table 30) to that produced by the mixed ovalbumin and water-in-oil emulsion (table 44).

b) Another explanation could be that there is an increased intake of antigen into the reticulo-endothelial system when tiny oil drops are present at the same time as the antigen.

Sbarra et al. (1962) showed that substances in solution and not normally phagocytosed, could be taken in by macrophages and polymorphs whilst these were engulfing solid particles. They called this 'piggy-back phagocytosis'.

c) A third possibility is that the antigens become adsorbed to the drops of oil and remain present for some time in the body as a sort of depot. Boroff and Tripp (1947) devised an agglutination test using an emulsion of olive oil droplets which were coated with streptococcal protein antigens. These aggregated and rose to the top of the tube if antibody was present. The author was unable to obtain an effect of this type with a mineral oil emulsion and ovalbumin. Other attempts which were made to adsorb or dissolve ovalbumin in oil did not indicate that any association occurred (experiment D4, page 260). This explanation cannot therefore be sustained.

d) Lastly, there may have been a mechanical cause for the enhanced titres. It was noticed on microscopical examination that the tiny droplets in oil-in-water and in multiple emulsions had a tendency to stick together or agglutinate without coalescing. Antigen trapped between the

droplets and subsequently acting as a slow release depot might be sufficient to account for the adjuvant effect, particularly the short-term one given by the oil-in-water emulsion (fig. 57).

On balance it was concluded that the components of the emulsion, when not actually enclosing the antigen, did not stimulate antibody production.

However, both oil and emulsifier had to be present if the antigen included in the mixture was to be efficiently used. Figure 60 shows that no detectable antibodies appeared if dry ovalbumin was incorporated in oil alone, though a normal type of adjuvant antibody response was seen if Arlacel was also present. That this was not merely a gross solution of ovalbumin in the Arlacel is shown by the fact that the mixture left after removal of the solid by centrifugation, only gave a poor fading response.[1] A biphasic water-in-oil emulsion is therefore not required for adjuvance, but the emulsifier plays a vital part in this type of mixture.

A strong argument against the theory that the only action of a depot is to act as an

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1. Of course a slight solubility of ovalbumin in Arlacel, which seems to be indicated here, may provide a route out for the main part of the antigen.

antigen store, slowly releasing its contents, is that removal of the depot does not abolish the adjuvant effect (Westwater, 1940; Holt, 1950; Freund and Lipton, 1955). However, it seems probable that secondary depots are rapidly formed all over the body very soon after a depot forming adjuvant has been inoculated. These can function in lieu of the main depot if this is removed (Freund and Lipton, 1955; Steiner et al., 1960; Weigle et al., 1960).

As a test of the effectiveness of tiny depots of this type, a very small quantity of a water-in-oil emulsion containing ovalbumin was injected into one lymph node. An adjuvant type of response followed which was appropriate in extent to the quantity of antigen injected. A central depot is not therefore necessary for adjuvance.

The chief observations leading to acceptance of the present theory

The key experiment in this study turned out to be one which had not been expected to yield any very useful results. Attempts to mimic the slow release action of a depot forming adjuvant have not generally been very successful. Holt (1950) gave thirty daily doses of plain diphtheria toxoid to guinea pigs, or an equivalent amount in

two doses of alum precipitated toxoid. The multiple dose group showed only about one fifth as much antitoxin as did the controls.

McKinney and Davenport (1961) found that there was a sort of threshold level of antigen (influenza virus) which had to be given daily before comparable titres to those shown after a whole dose in water-in-oil emulsion, were reached. As in Holt's experiment the total amount of antigen given over 42 days added up to the same dose as that given in adjuvant to the controls.

The use of fairly large daily doses of antigen by these investigators was in accord with the findings of Talmage and Dixon (1953). They reported that $^{131}I$ labelled antigens had a half life of about 14 days in a water-in-oil depot. This means that 99.9 per cent of the depot contents would be lost in 154 days. The present experiments do not confirm a short persistence of this order.

Mice inoculated with depots removed from other mice which had been given 2000, 400 or 100 µg of ovalbumin in emulsion 271 days before, showed quite good antibody responses (experiment D10, table 53). A rough check may be made of the probable antigen content of the material

injected into the second mice, by referring to the dose response curves for ovalbumin in a water-in-oil emulsion, figure 44. From this it can be seen that perhaps 10-200 µg of ovalbumin was present in the depot material given to each fresh mouse. This is equivalent to there having been between 5 and 50 µg of ovalbumin in each of the depots removed after 271 days.

A similar calculation for the ovalbumin content of depots transferred to three sets of mice (experiment D11, page 283) shows that perhaps 2 µg of ovalbumin was present in each depot at the third transfer after 544 days.

These figures correspond to a half life of ovalbumin in the emulsion of between 80 and 90 days. It is evident that if a short life of about 14 days was operative, the mice to which the depot contents were transferred could not have shown the antibody titres which they did. Talmage and Dixon calculated that after 301 days in a depot only 0.025 µg of antigen (bovine gamma-globulin) would remain from an initial dose of 50 mg when the half life was 14 days. There would have been much less left from the initial dose of 100-2000 µg used here. It may be that the emulsions used by Talmage and Dixon were not very stable, though they do report that the

rabbits inoculated showed a relatively constant antibody response for 301 days.

Some other workers have reported a long term persistence of antigen in water-in-oil emulsions. Halbert et al. (1946) and Herdegen et al. (1947) found antigenically active Shigella para-dysenteriae in water-in-oil emulsions for at least 22 weeks after injection. Most investigators, however, have not followed the animals over a long enough period for their results to be relevant here.

A consideration of these results indicated that the rate of release of antigen from a water-in-oil depot must be very slow. It was therefore decided that when an attempt was made to mimic the slow release mechanism, the total dose of antigen given should not equal the standard dose of 2 mg usually used for the experimental work in this study.

McKinney and Davenport (1961) showed that early release of antigen from a water-in-oil depot appeared to be important. They found that irradiation of mice up to 3 days after inoculation markedly lowered the peak antibody titres reached. Talmage and Dixon (1953) using $^{131}I$ labelled antigen found that there was a 20-30 per cent loss from the depot site during the first

24 hours.

A consideration of the mechanical effect of injection, the possible presence of large antigen drops or even of non-incorporated water phase in the inoculum initially, suggested that a large loss of antigen from the depot could occur shortly after injection. One experiment carried out, in which depots were re-injected into the mice from which they came and initiated a secondary response in them, seemed to confirm this (experiment D9, page 277).

Another result from the present work also seemed to show that a fairly large initial dose might be important. When very small primary doses of ovalbumin were given to mice, they did not appear to be sensitized to give a secondary response (table 57). Therefore a minimum initial stimulating dose might be required if detectable antibody levels were eventually to be raised. This is also in line with the concept that a minimum sensitizing primary dose of antigen is required before a secondary response can be elicited (Svehag and Mandel, 1964).

The initial inoculations were therefore made fairly large, but they were halved on each successive day until only 1 µg of ovalbumin per day in saline was being given to each mouse

(table 58). Over the 50 daily injections a total of only 171 µg of ovalbumin was given to each mouse. If this had been given as a single dose in saline only a very feeble response would have been observed (cf. table 57).

In fact, the response seen was very similar to that given by 2 mg of ovalbumin in water-in-oil emulsion (fig. 67). Furthermore, as a crucial pointer to the importance of a continual supply of antigen in maintaining the high level of antibody response, an immediate fall started when inoculations were stopped on the 50th day.

Small daily supplies of antigen are therefore sufficient to produce an adjuvant type of response. There appears to be no need for the direct intervention of the emulsion forming materials themselves in this effect. The action of the oil may be merely to protect the antigen from destruction and the form of the emulsion is such that a slow break-down and consequent release of antigen occurs. There is no need for the adjuvant itself to leave the depot, a concept which has bothered other investigators in view of the fibrous encapsulation of the depot which occurs after a subcutaneous inoculation.

The release of antigen from a depot of this type probably occurs in a slow, even and orderly

fashion. Post-mortem examination of several groups of mice in which the water-in-oil depots had been left in situ for long periods showed them to have a stratified appearance (experiments B2 and D3, pages 221 and 259). This appears to be due to the gradual break-down of the emulsion allowing more oil to rise to the top (experiment D16, page 300). It is exactly matched by the break-down of a water-in-oil emulsion seen when it is left in a bottle on the bench (fig. 72). The antigen leaks out at the bottom and as this happens the oil layer becomes thicker on top. This is the same mechanism, but upside down, to that seen when a glass of beer is poured out. The air-in-beer emulsion at the top gradually breaks down and the level of liquid in the glass rises.

Confirmatory evidence for the theory from other experiments

The results discussed above led to the conclusion that the adjuvant effect of a water-in-oil emulsion was solely due to a slow release of the protected and enclosed antigen over many months. Most of the results of the various experiments which had been carried out could now be seen to fall into the logical pattern to be expected from this theory.

Ovalbumin adsorbed on to aluminium hydroxide and given to mice by subcutaneous inoculation elicited similar titres to ovalbumin in water-in-oil emulsion. Aluminium hydroxide would be expected to work in rather the same way as an emulsion, by slowly releasing antigen. Figure 63 shows that the response curves for both were very similar.

When secondary inoculations of antigen in saline were given to mice already responding to the antigen in emulsion, an immediate rise in titre was produced. This later dropped back to the plateau level that would have been seen had no secondary injection been given (figs. 46 and 47). The secondary response was but an incident on the normal adjuvant response curve. This would be expected if in fact the plateau level was due to the continual slow release of antigen from the depot. It may be objected that this slow release itself constitutes a secondary response and that this is incompatible with a later one. However, D'Antona and Piazzi (1956) showed that the height of the secondary response is dependent on the dose given. The sudden arrival of a large amount of antigen might therefore be expected to cause a rapid increase in antibody production.

A multiple emulsion containing ovalbumin when given subcutaneously produced an excellent response rather higher than that given by a water-in-oil emulsion (fig. 54). Although the droplets in the emulsion originally injected were very tiny, rather larger ones were seen at post-mortem (table 37). However, when a multiple emulsion containing ovalbumin was given intravenously there was a rapid and high immediate response which was not sustained (fig. 56). In this case the tiny droplets injected would be dispersed separately through the various organs and could not fuse to form depots of a reasonable size. Each tiny drop would lose its contents quite quickly so that there would soon be no protected antigen left.

On the other hand, this rapid response may explain the higher titres found when antigens were given in multiple emulsion, compared to those for similar doses of antigen in water-in-oil emulsions (figs. 43 and 54 and table 38). Some of the tiny drops from a subcutaneous inoculation are bound to find their way into the blood stream and so cause this type of very rapid stimulation of the antibody forming cells. The remainder will coalesce and settle into a depot, releasing antigen gradually. The high initial

titre would then be expected to fall gradually to a plateau value as is seen in figure 54.

Antigen presented in multiple emulsions prepared with Tween gave higher titres than antigen in water-in-oil emulsion (tables 34, 35 and 38). When a multiple emulsion was made up with gum, the results were not so good (fig. 55). At post-mortem examination the depots formed by this material were found to consist of a hard gum matrix enclosing the emulsion droplets. These enclosed droplets might break down to release antigen as fast as they normally would have done, but any antigen that was released would reach the outside of the depot only slowly due to the gum covering. There would thus be less antigen to stimulate the antibody forming apparatus than that coming from a normal depot. This would fit in well with the lower, falling titres recorded.

The experiment in which methylene blue was incorporated into an emulsion showed that there really was a delayed release of material which had been incorporated into a water-in-oil emulsion. The method was not sensitive enough to show the very slow rate of release that it is necessary to postulate occurs if sufficient antigen is to remain available for titres to be kept up over long periods.

The experiments carried out to see whether ovalbumin could pass across the oil/Arlacel barrier were only successful in one case which is of doubtful significance (experiment D5, page 267). It was also impossible to detect any marked association, solution in, or adsorption of the ovalbumin to the adjuvant components. Even when a standard dose of ovalbumin was enclosed in oil any antibodies produced to it were at an undetectable level (fig. 60).

This suggests that the emulsion formulation is important for the production of adjuvance. When the emulsion is prepared with mineral oil the break-down is slow and there is a gradual release of very small quantities of antigen. The disperse phase then settles out to form a stratified layer from which antigen is liberated in relatively constant quantities over a long period of time.

Adjuvance was also seen when ovalbumin was enclosed in oil/Arlacel without a water phase. This mixture seems to have the ability of enclosing within itself droplets of water to make an emulsion (experiment D5, page 267 ). Smoky swirls of droplets are seen when it is put in the wells of an agar plate. It seems very probable that ovalbumin was able to leak out of such a

mixture much more easily than it could from completely inert mineral oil. There may also have been some slight solution of ovalbumin in the Arlacel as suggested above.

Diluting a standard water-in-oil emulsion containing ovalbumin with more oil/Arlacel did not affect the antibody response to it (fig. 59). The extra oil might have been expected to hamper water phase droplets from reaching the outer surface of the depot where they could release their contents and so reduce the final titre. However, if the stratifying process (fig. 72) took place fairly rapidly these depots would present the same picture as did those containing a full dose of antigen, later on in the adjuvant response. To judge by the antibody titre plateau seen, a constant antigen release was taking place. If break-down and consequent antigen release was only occurring at the bottom of the drops, the degree of dilution used would not be expected to have much effect on the rate of release. Later in the response a difference might have been seen for where there was less antigen in the depot this would be exhausted earlier and the antibody level would then drop. Unfortunately the experiment was not carried on for long enough to see whether this did in fact

occur.

The more dilute materials give a rather higher titre though this is not statistically different. This slight difference may have occurred in a similar fashion to the higher titres seen with multiple emulsions. The more dilute material will have a lower viscosity so that some of it may have broken away during the initial inoculation to give a more immediate stimulation. From other results it seems unlikely that the mere presence of more oil/Arlacel could account for the increase.

McKinney and Davenport (1961) considered that an initial release of antigen over the first few days was of importance in producing the adjuvant effect. The presence of antibody at this time might therefore be expected to have some effect on the shape of the curve. When this was tried, no difference was seen between the response of mice given anti-serum at the same time as ovalbumin in water-in-oil emulsion, and the response of mice not given serum (fig. 64). The experiment itself was of course not entirely satisfactory as an arbitrary single dose of anti-serum was used.

If the antigen initially released was not important to the adjuvant response its removal or

neutralization by antibody present at the time of inoculation would not alter the basic shape of the response curve. As mouse gamma-globulin only has a half life of 2 days (Dixon et al., 1952) the curve would be moved to the right a little, an effect which would not be readily seen. Insufficient anti-serum may also have been given. The picture is not entirely clear for antigen-antibody complexes can be good immunizing agents (Hartley, 1925). Also, if the adjuvant response curve is due to the slow release of antigen this antigen must combine with antibody very soon after it is released from the depot. But in some way it still stimulates a high titred plateau response.

It was unfortunate that it was not possible to detect any IgM type antibodies during the adjuvant response. Bauer et al. (1963) found that in addition to the known rise and fall of this type of antibody during the primary response, an additional rise occurred (in rabbits) when a secondary stimulation was given. Mice are known to show a similar antibody pattern at the primary response (Berlin, 1963). The present experiments only doubtfully detected any sign of this, probably because the serum collection was not started until the IgM antibody peak had passed.

More disappointing was the failure to show any antibody of this type after a secondary response. This failure leaves open the question of whether the production of such an antibody is stimulated by a continual release of antibody from the depot, which in effect must constitute a continual secondary response. On the other hand, the mouse may not show a similar IgM antibody rise after secondary stimulation as does the rabbit, or its extent may be proportional to the dose given.

A remaining experiment is slightly difficult to explain. In this the depots were re-injected into the mice from which they had been removed (experiment D9, page 277). These mice showed a secondary response. Holt (1950) carried out a similar experiment using guinea pigs which had been inoculated with diphtheria toxoid which had been adsorbed on to alum. He obtained a similar result and took this to mean that after 14 days the depot contents were so walled off as to be incapable of taking further part in the adjuvant response. The response curves for a single injection of the precipitated toxoid were of a very peaked shape in comparison to the slow rise followed by a plateau shown by the mice given ovalbumin in water-in-oil emulsion, so there was

some reason for this view.

In the present study it is probable that the mechanical disturbance of the emulsion at injection was sufficient to cause a certain amount of break-down. Small dispersed particles of emulsion may also have been produced. Due to their greater total surface area the release of antigen from these would have been quite large and sufficient to cause a marked secondary stimulation. D'Antona and Piazzi (1956) showed that the size of a secondary response is related to the dose of antigen given. The visible response seen here thus only indicates that much more antigen has been released at one time rather than that continual release from the depot does not occur. The fact that a secondary response is seen after re-injection of the emulsion is a further confirmation of the findings of other workers that a large amount of antigen is lost from the depot soon after inoculation.

Some implications of the proposed theory of the action of water-in-oil emulsion adjuvants

If the sole reason for the adjuvant action of a water-in-oil emulsion is its ability to hold, protect, and very slowly release the antigen, this has important theoretical and practical implications.

From the theoretical point of view it is of interest that the extremely tiny quantity of antigen released, which must combine with antibody as soon as it leaves the protection of the depot, is so efficient in stimulating the antibody forming mechanism. Rapid elimination in an inactive state might be expected (Weigle, 1961). It would be instructive to attempt to reproduce an adjuvant type of response with tiny doses of an antigen-antibody mixture given daily as in the key experiment described above.

For the practical use of this type of adjuvant in immunization procedures, a consideration of the mechanism proposed to account for the slow antigen release, is important. The stratification effect seen in the mouse depots occurs because mice always remain one way up. This means that the depot contents settle into a stable geometry and an even rate of emulsion break-down and antigen release follows.

It is difficult to foresee whether a depot which is being shaken about all the time as in a man's arm or in the neck of a fowl will break down regularly in this way. The rate of antigen release may be slower or faster or irregular and an investigation of this effect is required. Careful thought should also be given the most

suitable siting in the body for this type of adjuvant depot.

The intramuscular inoculation of a water-in-oil emulsion was found by Salk et al. (1952) to eliminate the troublesome oil cysts and other reactions seen in man after subcutaneous injection. This practice is followed in the present-day use of these adjuvants with influenza virus vaccine. Salk and Laurent (1952) reported that in monkeys there was a dispersion of intramuscularly inoculated emulsion as tiny droplets along the muscle fibres.

A multiple emulsion is prepared in the form of tiny drops. When this material is given intravenously the response is short and quickly falls (fig. 56). The desired long term response is not obtained. It was postulated above that this is because the tiny droplets break down fast due to their large total surface area and no lasting depot is formed.

Water-in-oil emulsion given intramuscularly or multiple emulsion given subcutaneously will not form quite such small isolated droplets, but the rapid exhaustion of the antibody content of small drops may explain the fall seen in the titres of human volunteers given influenza vaccine intramuscularly in water-in-oil emulsion.

A more long lasting plateau titre of the type seen in mice would have been desirable. Arm movement causing a mixing effect in the tiny depots may also have played a part in this.

The present study was carried out with a soluble antigen of small molecular weight. Many bacterial and viral antigens will be much larger than ovalbumin. Even after release from the emulsion these antigens may not be able to leave the encapsulating fibrous tissue surrounding the depot to exert their stimulating effect. This may be the reason for some of the disappointing results which are understood to have occurred with viral antigens in water-in-oil emulsions.

On the other hand, the toxic fractions of some microbial and viral antigens may give them a centrally acting adjuvant action which may overlay the effect of the emulsion. This will be particularly evident if long-term investigations of the antibody response are not carried out, and the point is not reached at which the plateau level of antibody produced by the long-term slow release effect becomes evident.

If antigen size is the only difficulty, break up of the organism followed by extraction of the wanted antigen in its purest form may be useful. This work may also be important to

stabilize the antigen. If the depot is to be of long-term value it is essential that the contained antigen is very stable under these conditions.

Acting in the way suggested, the water-in-oil emulsion adjuvants are a most elegant slow release mechanism ideal for their purpose. By altering their viscosity, stability, and form by technical means and by choosing a suitable site for the inoculum, it should be possible to devise practical adjuvants to meet all the immunization needs of man and the domestic animals.

B. The use of mineral oil adjuvants in practical immunization procedures

Mice inoculated with a single dose of ovalbumin enclosed in a water-in-oil emulsion gave a peak antibody titre which was about one thousand-fold higher than that given by mice inoculated with the same quantity of ovalbumin in saline (tables 26 and 30). Not only was a very high titre reached, but, more important, it was sustained at this level for at least a year (fig. 54). The usefulness of any adjuvant which can elicit a response curve in this form is self-evident. Attack by micro-organisms is sporadic and unexpected; active immunity cannot be supplied instantly when required, so that immunizing procedures with a long-term validity are the most valuable.

Influenza in man is an excellent model of the patterns of disease against which protection is needed. Some strains are always present and are able to attack here and there in a community without warning. A major mutation has the characteristics of a new disease; innate immunity is wanting everywhere and the virus rapidly spreads over the world documented by international organizations.

For protection against influenza virus in

the first form, a long duration of immunity produced by a simple, single dose means is required. To defeat its spread in the pandemic state, a speedy production of vaccine ahead of its arrival is necessary. The mineral oil adjuvants appear to be valuable in both roles. Not only do they give the long lasting protection required, but the enormous increase in antigenic efficiency which they engender would enable smaller quantities of a precious new strain to be effective as an immunizing agent.

The antibody response of men to saline or water-in-oil emulsion influenza virus vaccines

When, in the present study, an influenza virus vaccine was tested in man it was not possible to plot many points on the response curve. The general shape of a complete curve may be gauged by examining the two curves in figure 73. The antibody level against the A2 strain of virus in the laboratory staff group at 8 months, falls exactly on the line drawn between the 4th and 12th month titres of the student group. A smoothed curve would show that the peak titre of each group was reached at about the second month. At the 4th month the students given the vaccine in adjuvant show about five times as much antibody as do those given the

saline vaccine (table 64).

The adjuvant vaccine only contained one-fifth as much antigen as did the saline vaccine, but even when this is allowed for, the adjuvance shown is very much less impressive than that seen with ovalbumin in mice. In addition, the persistence of titres at a high level which is such a feature of the response of mice given ovalbumin in adjuvant is not marked. In the student group, antibodies to both viruses appear to fall off at the same rate irrespective of whether the antigens were given in saline or in adjuvant emulsion. The adjuvant group show an advantage but this is due to the higher peak titre reached.

The response of men to influenza virus vaccine in a water-in-oil emulsion is therefore dissimilar to that given by mice in the ovalbumin model. The use of adjuvant was advantageous as it stimulated the production of much more antibody with less antigen, but the peculiar persistence of titre which is so desirable was not seen.

Several reasons for this lack of a high persistent titre may be suggested. In discussion of the mode of action of water-in-oil emulsion adjuvants the belief was expressed that the sole

reason for the action of these adjuvants is their ability to hold and protect antigen which is then gradually released over a very long period of time. The oil emulsion is otherwise quite inert. When mice were given a water-in-oil emulsion subcutaneously it formed a circumscribed pea-like depot which could be easily seen and palpated (table 39). The human volunteers were given the emulsified vaccine intramuscularly so that single depots of this type were not formed. This latter route causes less reaction and the oil emulsion becomes dispersed amongst the muscle fibres (Salk and Laurent, 1952). It seems probable that these small droplets will each exhaust their supply of vaccine in a shorter period than will a single large depot. As the plateau titre seen in mice is probably maintained by the slow continual release of antigen, this titre will fall as exhaustion of the antigen contained in the droplets approaches. It may be that this effect is also seen in the falling curve for the response to ovalbumin in a multiple emulsion shown in figure 54. When injected subcutaneously the multiple emulsion tended to form a dispersed depot of small droplets (table 37).

Another explanation may spring from the fact that a whole micro-organism rather than a soluble

antigen of small molecular dimensions is being used in the vaccine. Though the virus may be released easily from the oil drops it may not be able to escape from the encapsulation surrounding the depot. There is some evidence that encapsulation of oil drops occurs even when the emulsion is given intramuscularly (Peck et al., 1964).

It is interesting to speculate on other possible reasons for the relatively poor results seen. Any soluble antigen released will at once be in an environment of gross antibody excess; all the antigenically active sites on it will rapidly be occupied by antibody molecules so that no aggregation of the particles will occur and the neutralized antigen will remain of small dimensions. On the other hand, a complex virus particle liberated whole from the emulsion may meet many different antibodies which can react with it. Not only will it be coated with these, but some of them may be present in such low concentration that an aggregation between several particles is a possibility.

Quite apart from the difficulty that particles affected in this way may have in leaving their environment, the pharmacological activity of such complexes of many different antigens and antibodies may lead to destruction of the antigen

in a way that does not stimulate further antibody production (Weigle, 1961). Only soluble fragments of the virus particles may be effective in the manner desired, but some of these may be caught up in the reactions occurring round the complete organisms. It is also possible that the virus may become attached to the fibrous capsule or trapped blood cells by its haemagglutinin. As the vaccine has been formalinized, its ability to subsequently elute from this will have been destroyed (Rhodes and van Rooyen, 1962).

On the other hand, it may be that few of the responses of man to influenza virus vaccine were primary responses as were those of the mice in the model experiments. Many of the volunteers must certainly have already been antigenically stimulated by virus of the strains used in the vaccine.

It was noted earlier that the results of the titration of antibody against the A2 strain of virus are closely comparable in both the student and laboratory staff groups. There is, however, a divergence in the levels of antibody against the B strain of virus. The laboratory staff show a much higher titre at 8 months than that which can be estimated from the graph for the student group at that point. The staff titres

are in fact higher at 8 months than those of the students at 4 months. This suggests that many of the laboratory staff are showing an enhanced response to the B virus because they have met it before, whereas few of the students have been infected with this strain.

The student group consisted of volunteers aged between 20 and 26 years whereas the laboratory staff group were aged from 21 to 59 years (table 63). Some older members of the staff group might therefore have had a different experience of influenza viruses than the young students. However, table 66, which shows the titres of antibody against B virus for the different age groups in the staff group, does not indicate that there was any difference in response between the young and old volunteers.

On the other hand, the difference in response to the B strain of virus by these two groups may be geographically determined. The laboratory staff could have been sensitized by an Edinburgh epidemic which occurred whilst the students were dispersed before coming up to the University.

If all or most of the volunteers are presenting a secondary response a full curve might then resemble those in figures 46 and 47.

In the experiments to which these figures refer, inoculations of ovalbumin in saline were given to mice already responding to the antigen in a water-in-oil emulsion. Secondary responses were seen but these soon fell back and the titres stabilized at the normal plateau titre seen after a single injection of ovalbumin in water-in-oil emulsion. It may be that a similar stabilization at a low plateau titre would have been seen here if enough points had been obtained for the graph and the experiment had been continued for a longer period.

Another complication may arise from the nature of the antigen. The influenza virus is complex with a lipid coat and toxic properties (Rhodes and van Rooyen, 1962). This may cause some stimulation of the reticulo-endothelial system in the manner of the lipopolysaccharide endotoxins (Farthing, 1961). In effect, the virus may contain a built-in adjuvant. If a mechanism of this type is active, the action of the whole vaccine will be complex and difficult to interpret.

It seems probable that this slightly disappointing response to influenza virus vaccine given in water-in-oil emulsion is due to the heterogeneity of the experimental conditions of

this study. The presence of a secondary response in some of the volunteers, together with some adjuvant properties of the virus itself may have enhanced the response to the saline vaccine more than that to the adjuvant vaccine. The steady rate of decline of the titres does, however, suggest that early exhaustion of the depot due to its intramuscular siting may be of considerable importance.

Advantages shown by the adjuvant form of the influenza virus vaccine

The results obtained in this trial of an adjuvant influenza virus vaccine in man did not show the dramatic advantage over the saline vaccine that experiments with ovalbumin in mice had led one to expect. However, the results do not indicate that the adjuvant formulation was valueless; in fact they show that this method of presenting the antigens has certain advantages.

The adjuvant form of the vaccine contained one-fifth as much antigen as did the saline vaccine but the response to it was very much higher. This in itself may not be very important as there is some evidence that very low titres of haemagglutination inhibiting antibody (1:16 or below) are sufficient to protect against natural infection (Meiklejohn et al., 1952).

There also seems to be no doubt that influenza vaccines containing antigens prepared in the same way as those used here are protective (Hawkins et al., 1956; Meiklejohn, 1962). This being so, the volunteers given the adjuvant vaccine will be protected for a longer period. They had the higher titres and the graphs show that the titres are falling at the same rate in the case of each vaccine.

The advantage of the adjuvant vaccine is well illustrated in figure 74. This shows histograms of the fold changes in titre between the initial, pre-vaccination, antibody level and that measured 1, 4, and 12 months later. A line has been drawn between the 2- and 4-fold levels on each histogram; to allow for experimental error it is assumed that at least a four-fold rise in titre must be recorded before a subject can be said to have responded to the vaccine.

Consider the results for the A2 strain of virus. The diagrams show that a year after vaccination nearly all (95 per cent) of those given adjuvant vaccine are still showing titres well in advance of their resting levels before vaccination. Though 84 per cent of the group given the saline vaccine are still over the line at this time, it is apparent that many of these

will soon fall back to their pre-vaccination level of protection within a few months.

Results for the B strain of virus are not as striking, though it is evident that the advantage lies with the adjuvant formulation.

When considering any results from trials with influenza vaccines the question of who the protection should be aimed at must be considered. The virus strains used in this particular vaccine are those to which the population may have already been exposed. Many of the subjects inoculated will have already been sensitized and be showing a secondary rather than a primary response. The titres of these volunteers make the best showing on the graphs and charts but they are probably already protected.

A minority of the population will not have met the antigens, will give a poor, primary type response but may end up with protection where they had none before. A saline vaccine would be expected to provoke a primary and probably poor response in these subjects whereas the adjuvant formulation, with its built-in secondary stimulation mechanism, might enable them to show much better final titres.

A count of the fold changes in titre for students whose initial antibody titres were high

or low shows that those with low initial antibody levels responded better than did those with initial high antibody levels (table 65). However, the pattern is the same for both vaccines so the adjuvant vaccine does not show any superiority. The reason for this may be that the virus itself is slightly adjuvant and the response is always better than that seen with simple antigens.

The reactions of man to the inoculation of a water-in-oil emulsion vaccine

Except when an epidemic threatens due to a new mutation of the virus, influenza vaccine will not be readily accepted by the general population unless it is very easily administered and causes no noticeable reactions. This is especially important in the case of children who are probably an important factor in the spread of influenza virus during an epidemic (Hennessy et al., 1964).

The first oil emulsion influenza vaccines to be used caused most unpleasant oil cysts and abscesses (Henle and Henle, 1945; M.R.C. Report, 1957). The work of Salk and his colleagues showed that these side effects could be eliminated by choosing tested materials and by giving the vaccine intramuscularly. McLean et al. (1964) found that Berlin's mouse test (1962) was an

excellent screening method to check completed emulsions for safety. Only those which were shown to be unsatisfactory by this test caused nodules or abscesses in man.

In the present study, records were kept of the reactions encountered with both of the vaccines (table 67). Though these appear to be numerous it should be made clear at once that no oil cysts or any reaction of great moment was reported. The information was collected by specifically questioning each subject. This has undoubtedly emphasized the occurrence of reactions, but the fact that they were remembered indicates that they were not negligible.

The few general reactions were reports of slight malaise following inoculation and these might or might not have been actually associated with it. The toxic properties of the virus and remaining egg-derived materials present in the inoculum would be expected to show their effects most markedly in the saline group. Incorporation into a water-in-oil emulsion would undoubtedly reduce their toxic effect (Ramon et al., 1937; Halbert et al., 1945). The distribution in the table shows no such bias, so it is probable that most of the reports are of chance indisposition not related to the vaccination

itself.

Reports of pain show a distribution which may be related to the route of injection used. In all cases it was very slight. Minor lesions consisted of erythema, induration and swelling persisting for several days and were almost entirely confined to the saline group. These lesions were not usually noticed until the area of redness was brought to the volunteer's notice by someone else.

Despite the frequency of mild reactions almost all the subjects found both vaccines quite acceptable. On the whole, the adjuvant vaccine was rather better received, some subjects having found the swelling and erythema produced by the saline vaccine slightly alarming. This latter effect would, of course, be reduced if a non-toxic vaccine prepared from purified viral haemagglutinins was used (Davenport et al., 1964).

The most detailed recent account of a trial of a water-in-oil emulsion vaccine is that of Cutler et al. (1962). They used a poliomyelitis vaccine (which, of course, had none of the known toxic attributes of influenza virus) incorporated into an emulsion prepared from the same materials as those used in the present trial. Only 1.1 per cent of 23,000 recipients showed any side effects.

These consisted almost entirely of slight local pain and tenderness. Bell et al. (1961) also found only 0.03 per cent (in Caucasians) to 1.47 per cent (in Negroes) of cysts or abscesses in 11,000 people given an oil adjuvant influenza vaccine. The safety of this type of adjuvant thus seems to be well established.

The effect of 'booster' inoculations and the use of the same adjuvant with other vaccines given later

With many vaccines it has become almost a tradition to follow an initial dose with a 'booster' some weeks later. When saline vaccines are used this is undoubtedly valuable especially where no immunity is present initially. The oil adjuvant vaccines, on the other hand, should produce a high and long-lasting antibody level sufficient to prime the defence mechanism for a long period. The value of increasing this titre at all is debatable and the efficiency with which it can be done, unknown.

Using the model system of mice given ovalbumin in a water-in-oil emulsion, some tests were carried out to elucidate this point. Mice already responding to the antigen, which had been inoculated in a water-in-oil emulsion, were given a single 'booster' secondary injection of oval-

bumin in saline. Injections given on the 5th and 10th days, whilst the climb of the titre to plateau level was occurring, had no effect on the shape of the curve (fig. 45). Inoculations given later, on the 20th, 40th, 80th or 129th days, produced typical secondary responses (figs. 46 and 47). These appeared to be very similar to one another (fig. 48). In each case the post-secondary fall in titre continued fairly rapidly until a level was reached which was equivalent to the plateau of response which would have been seen had no secondary inoculation been given.

Though a short-term advantage was obtained the long-term picture was not altered. If lasting protective antibody levels are required, 'booster' inoculations appear to be unnecessary. Whether this would be so with the rather different curve seen with influenza virus vaccine in man is not known. To obtain a higher peak titre might mean that it would fall faster so that there would be no advantage. If it fell at the same rate, the time during which protective antibody titres were available would be extended.

It was also evident from the experiment that the antibody forming mechanism was still as capable of responding to extra antigen later in

the response as it was at the beginning. 'Tiredness' did not occur due to the continual high level of antibody being produced.

If water-in-oil emulsion vaccines come into general use, man and animals may be given a number of different vaccines formulated in this way during their lives. The oil component of these emulsions is not metabolised and will probably persist for many years. It is therefore important to know whether there is any mutual interaction between two vaccines of this type which is due to the emulsion forming materials employed.

Mice responding to an antigen which had been given in water-in-oil emulsion were therefore inoculated later with another, unrelated, antigen in the same type of adjuvant. The results, illustrated in figures 49-52, showed that neither vaccine had any effect at all on the response to the other. It is therefore assumed that it would be perfectly satisfactory to formulate many vaccines in this manner and inoculate them as required.

A new method of improving the convenience of water-in-oil emulsions for practical use

The adjuvant influenza vaccine used in the tests with human volunteers ('Admune') was packed

in cartridge inserts which were used in a dental syringe. This inoculum was given through a needle with an outside diameter of 0.75 mm. The trauma caused by the use of a needle of this size undoubtedly contributed to the reports that pain at the site of injection lasted longer in this group.

Water-in-oil emulsions are thick, creamy materials difficult to pour or inject through anything but a wide bore needle. Even with the powerful syringes used, small quantities of emulsion were left in the cartridge on a few occasions. For human use, single dose syringes or cartridges are to be recommended but for many veterinary applications this is completely uneconomic. Unfortunately, water-in-oil emulsions are unsuited for use in multi-dose containers. To fill any syringe with these materials, especially without entrapping air, is tedious and difficult.

By preparing a multiple emulsion from the water-in-oil emulsion many of these difficulties may be overcome. The thick unpourable vaccine is converted into a mobile water-like fluid which can easily be drawn up into a syringe and injected through a fine needle. A vaccine formulated in this way will be at least as

effective as one in a water-in-oil emulsion (figs. 43 and 54 and table 38). There is also some evidence (reviewed in the discussion on the mode of action of adjuvants) that the response to an antigen contained in a multiple emulsion may occur more quickly and earlier due to a rapid entry into the general circulation of tiny drops containing portions of the antigen.

Other advantages are, that large single depots of unmetabolisable oil are not produced, and that it may be possible to enclose an antigen in less oil whilst still retaining a total dose volume which can be simply injected with an ordinary syringe.

This new formulation may therefore considerably extend the range of employment of water-in-oil emulsion vaccines.

## Parts Seven and Eight

## SUMMARY, ACKNOWLEDGEMENTS, and REFERENCES

## SUMMARY

A review has been made of the literature relating to:

(a) Adjuvants in general, the oil emulsion adjuvants in particular, and the mode of action of the depot-forming adjuvants.

(b) The preparation and purity of the antigen, ovalbumin, which was used in the chief investigations.

(c) The tanned red cell agglutination test, the antibody measuring method employed in this study. Special reference was made to the effect of conalbumin on the specificity of the test when used to measure antibodies raised against impure preparations of ovalbumin.

It was found that commercially obtained twice recrystallized ovalbumin, when examined by immunological methods, contained at least four other antigens in addition to ovalbumin. Conalbumin was one of these antigens. Egg-white proteins eluted from CM-cellulose were characterized immunologically. It was found that very pure, conalbumin-free ovalbumin could be prepared by this technique.

From this a method was developed for the preparation of highly purified ovalbumin in

quantity for use as an antigen. Ovalbumin was first extracted from egg-white by an abridged method of crystallization with ammonium sulphate. The remaining impurities were then removed by a simple chromatographic process on a CM-cellulose column.

A suggestion that tanned cells coated with impure ovalbumin would respond chiefly to traces of anti-conalbumin in an anti-ovalbumin serum was not confirmed. Cells coated with a mixture of ovalbumin and conalbumin responded to whichever antibody was present in the greatest quantity in the serum. Conalbumin appeared to be a very good antigen in the rabbit and it was concluded that this may account for the anomalous results reported by other workers.

The conditions under which formalinized sheep red cells are tanned and coated were examined in detail. Wide variations in antigen concentration, pH, temperature, time, materials, etc., had little effect on the resulting sensitivity of the cells. Variations in the strength of the tannic acid used to treat the cells had a marked effect. A method was devised for tanning and coating formalinized sheep cells in bulk. The cells were then stored frozen in small portions for use as required over many

months.

Ovalbumin inoculated into mice subcutaneously in a water-in-oil emulsion was found to stimulate a characteristically high and sustained response. A high 'plateau' antibody titre was seen which lasted for at least a year.

Secondary inoculations of ovalbumin in saline given to mice on the 5th and 10th days after their inoculation with ovalbumin in a water-in-oil emulsion had no effect on the response curve. Secondary inoculations given between the 20th and 129th days stimulated secondary responses which appeared as transitory incidents on the general pattern of the adjuvant response. These secondary response curves were almost identical in shape. It was concluded that 'booster' inoculations may not be found useful when an adjuvant vaccine of this type is used.

Two unrelated antigens, ovalbumin and human-gamma-globulin, given separately in water-in-oil emulsions to the same animals at different times showed no mutual interactions. From this it was concluded that several vaccines to be given at different times could be satisfactorily formulated as water-in-oil emulsions.

The emulsifiers Arlacel A and Tween 80

showed no adjuvant activity when used alone. Complete water-in-oil emulsion when inoculated into mice apart from the antigen in time or place, had no adjuvant effect. Ovalbumin inoculated in an oil-in-water emulsion stimulated slightly enhanced titres but no sustained response. It is suggested that this response is due to mechanical reasons or 'piggy-back' phagocytosis. No association between, or adsorption of antigen on to the components of the emulsion could be demonstrated. IgM type antibody production was not detected during the adjuvant response.

A tiny volume of water-in-oil emulsion containing ovalbumin and injected directly into a lymph node, stimulated an adjuvant response. This is held to confirm the view of other workers that small dispersed depots can be important as sources of antigen. Removal of the main antigen depot at the injection site is not therefore a good test of the ability of a depot-forming adjuvant to act by slowly releasing antigen over a long period of time.

Ovalbumin was shown to persist in a fully active form within an emulsion <u>in vivo</u> in the mouse for at least 544 days. The break-down of water-in-oil emulsions <u>in vitro</u> and <u>in vivo</u>

appeared to follow a similar course. In both cases there was a slow release of the water phase and hence of the antigen.

The adjuvant type of response curve in mice could be mimicked by very small daily doses of antigen in saline. It was therefore postulated that the only action of a water-in-oil emulsion adjuvant is to hold, protect, and then very slowly release the antigen into the circulation over a long period of time. The implications of this theory are discussed.

A new type of oil emulsion, a multiple emulsion in which the antigen is contained within a secondary dispersed phase in oil droplets is described. This is a mobile liquid, easily withdrawn from containers and used with a normal syringe. Its adjuvant properties were found to be at least as great as those of a normal water-in-oil emulsion.

The responses of men to commercial saline and water-in-oil emulsion influenza virus vaccines were compared. The adjuvant vaccine, though containing only one-fifth as much antigenic material as the saline vaccine, stimulated much higher titres. The titres in both groups were found to fall at the same rate and no persistent plateau titre was detected such as

that seen in mice given ovalbumin in water-in-oil emulsion. The reason for this is discussed and explanations suggested.

The reactions to the use of saline and adjuvant influenza virus vaccines in man were recorded. Reactions to the saline vaccine were more marked than those to the adjuvant vaccine but no serious reactions were reported. Pain and tenderness lasted slightly longer in the volunteers inoculated with the adjuvant vaccine, which was given intramuscularly, than in those given the saline vaccine subcutaneously. This was probably related to the routes of inoculation used. The volunteers found the adjuvant vaccine quite acceptable and it was concluded that the water-in-oil emulsion adjuvants are useful adjuncts to immunization procedures.

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## Publications from the contents of this thesis

Statement in accordance with Ph.D. additional regulation No. 13:

1. A paper by the author entitled 'Ovalbumin and conalbumin in the tanned-cell agglutination test' was read to a meeting of the British Society for Immunology on 22nd November 1963.

2. Multiple emulsion adjuvants are the subject of British patent application number 42210/63 'Improvements Relating to Injectable Compositions' filed by the National Research Development Corporation on 25th October 1963.

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